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VISCERO-CUTANEOUS COLLAGENOSIS

A STUDY OF THE INTERMEDIATE FORMS OF DERMATOMYOSITIS, SCLERODERMA, AND DISSEMINATED LUPUS ERYTHEMATOSUS

BY

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(RECEIVED FOR PUBLICATION SEPTEMBER 14, 1954)

It has been known for some time that dermatomyositis and scleroderma are closely related (Freudenthal, 1940; Dowling and Freudenthal, 1938) and that intermediate forms exist between these two entities. In the present paper further material will be presented in support of this idea.

It was pointed out in a previous paper (Pagel, Woolf, and Asher, 1949) that profound visceral changes, probably ischaemic, resulting from vascular lesions, are often found in dermatomyositis simultaneously with the skin and muscle changes. In scleroderma concomitant visceral and skin lesions have long been known and extensively recorded—so much so that the demand has now been made for the abolition of the name "scleroderma" and the substitution in its place of the term "progressive diffuse sclerosis" (Goetz, 1945; Beigelman, Goldner, and Bayles, 1953), a clumsier but more precisely descriptive name. Some of the changes seen resemble those observed in disseminated lupus erythematosus. It would thus appear that overlapping features are not limited to scleroderma and dermatomyositis, but include members of the group of so-called collagen diseases. It is therefore suggested that an even more general term should be used for these overlapping syndromes, "viscero-cutaneous collagenosis."

ILLUSTRATIVE CASES

In the present paper, based on illustrative cases, the following points are discussed in detail. (1) Transition of dermatomyositis to progressive diffuse sclerosis; (2) the histological changes in nail cuticle in dermatomyositis and disseminated lupus erythematosus, and their possible diagnostic significance; (3) suprarenal changes in progressive diffuse sclerosis and dermatomyositis, suggesting nodular fibrosis developing from focal fibrinoid necrosis; (4) transitional renal and muscular changes; (5) the muscular changes in dermatomyositis compared with myositis induced experimentally with muscular extracts.

Transition of Dermatomyositis to Progressive Diffuse Sclerosis

Case 1.—The patient, a woman aged 36, was originally described as Case 3 by Pagel, Woolf, and Asher. She developed dermatomyositis 11 years before death, and five years before death both legs had to be amputated, as severe chronic and atrophic sclerodermatous lesions had rendered them ankylosed and useless. For three years after the amputations she had intermittent acute attacks of urinary infection, for which she was admitted on several occasions, responding each time to chemotherapy. Four years after operation a radiograph of the chest showed large "cysts" in the lungs. She was admitted on January 19, 1953, with a history of acute left-sided pyelitis. She was dehydrated and drowsy and her blood pressure was 80 mm. Hg systolic. The blood urea was 163 mg. per 100 ml. Two days after admission she became cyanosed and shocked, developed rapid respirations, and died the following day.

Necropsy (No. 53/40).—The body was that of a woman looking older than her age, with scarring and pigmentation of the skin, particularly round the breasts and sites of amputation. The right lung gave a gross impression of pulmo lobatus, i.e., there were irregular deep fissures in both upper and lower lobes (Fig. 1) and on the cut surface a number of well-defined, grey, fibrosed areas, measuring about 2.5 × 1.5 cm., chiefly in the cortico-pleural, infraclavicular, and hilar areas. These contained systems of bullae up to 2 cm. in diameter (Figs. 2 and 3). The left lung was not adherent and contained one or two small, fibrous areas. Both lungs showed basal oedema and congestion. There were a few fine pericardial adhesions. The heart showed no appreciable changes.

The liver weighed 1,080 g. and was firm. The cut surface showed a fine reticular fibrosis throughout, with a loss of lobular pattern. The spleen weighed 225 g., the pulp was soft and purplish, with obscure Malpighian bodies. The kidneys were small, very irregularly scarred, with pale, mottled subcapsular surfaces, from which the capsule stripped easily. The cut surfaces showed good cortico-medullary

FIG. 1

FIG. 2

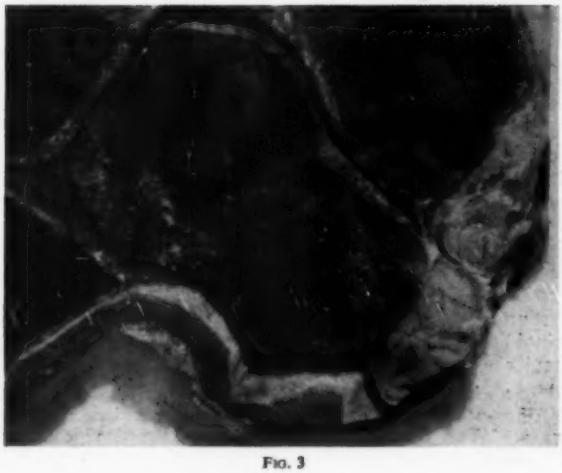


FIG. 3

FIG. 1.—Case 1: "Pulmo lobatus," pleural surface of right lung.

FIG. 2.—Case 1: Cut surface of right lung, showing an infra-clavicular area of fibrosis. Inset: A similar area in the lower, more dorsal portion of the right upper lobe, with cyst formation.

FIG. 3.—Case 1: another cut surface of right lung, showing pleural thickening, including interlobar fissures and apical and cortico-pleural areas of fibrosis with cyst formation.

FIG. 4.—Case 1: histological section through lung. Fibrinoid lining of dilated alveolar ducts (arrows) (×80). Inset: close-up of fibrinoid lining (haematoxylin and eosin, ×300).

FIG. 5.—Case 1: histological section of skin. Thinning of epidermis with sub-epithelial oedema and lymphorrhagia in the cutis (haematoxylin and eosin, × 80).

FIG. 6.—Case 1: liver. Histological section showing multilobular liver cirrhosis (haematoxylin and eosin, × 80).

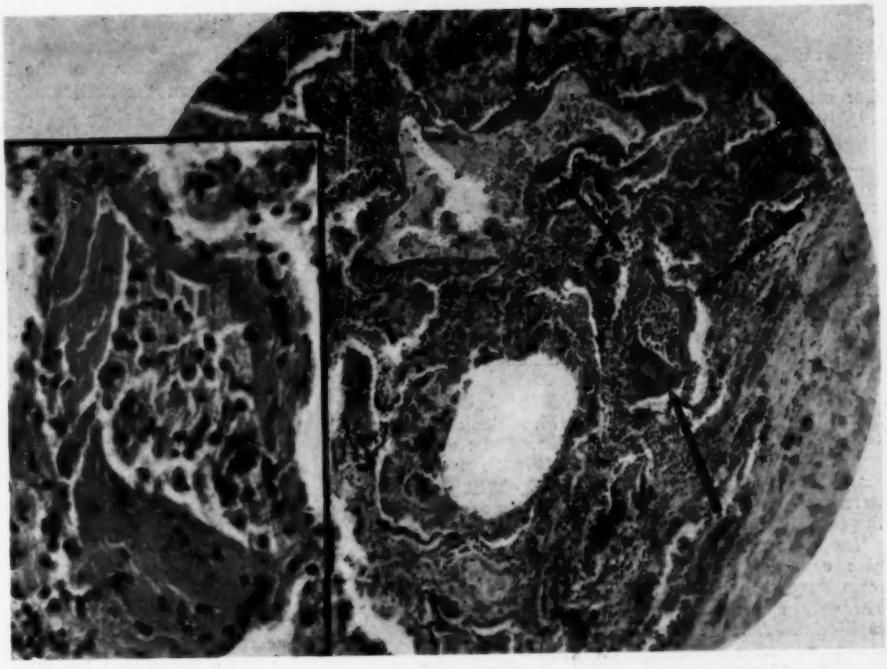


FIG. 4

FIG. 5

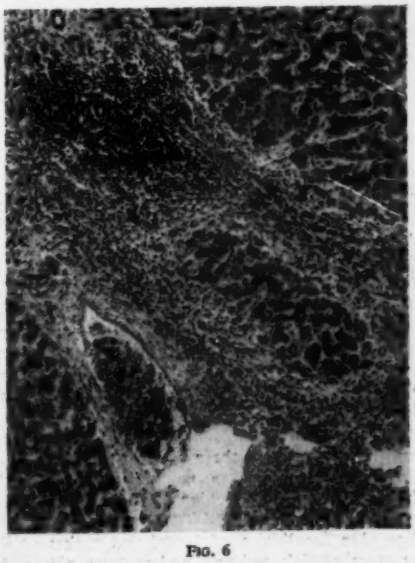


FIG. 6

demarcation, but the cortical thickness was reduced to 0.5 cm. The pelves contained purulent urine. Cortical lipoid in the suprarenals was reduced.

Histology.—In the lungs, sections through the triangular fibrous foci and the multiple subpleural scars showed the uniform picture of collapse-induration with compensatory emphysema. In the fibrous tissue, curly hyperplastic elastic fibres were seen in large numbers forming plaques, chiefly subpleurally but also evenly distributed throughout the collagenous areas. Additional features were fibrinoid lining of dilated alveolar ducts (Fig. 4), collapsed bronchioli, crystalline foreign bodies with foreign-body giant cells in collapsed alveoli, and concentric intimal thickening, with elastic reduplication, of the pulmonary arterial branches.

The skin from various parts of the body was markedly thinned in the epidermis with sub-epidermal oedema and conglutination of collagen. Some of the sections showed focal lymphorrhagia (Fig. 5). Irregular clumps and plaques of fragmented elastic fibres were scattered throughout the dermis, especially in the foci of conglutinated collagen.

The liver showed a multilobular portal cirrhosis with collections of lymphocytes in the periportal septa around hyperplastic bile capillaries (Fig. 6). There was a marked capsular fibrosis, containing bile capillaries. The parenchymal cells showed little change.

In the kidneys there were areas of hyalinized glomeruli with some tubular atrophy and marked focal lymphocytic collections in these places. Some of the glomeruli showed partially lamellated hyaline deposits, the rest of the Malpighian corpuscles being encrusted with nummular crystalline material. There were also marked eccentric and concentric intimal thickenings of arterioles with elastic reduplication (Fig. 7). Exceptionally, a small gap in the elastica was seen (Fig. 7). Some of the atrophic and dilated tubules contained granular and homogeneous casts. One isolated but very definite area of fibrinoid necrosis was seen in the stroma between a hyalinized glomerulus and some normal tubules. This area was strongly positive with the fibrin and periodic acid stains, and failed to stain with elastic-van-Gieson stain (Fig. 8).

The skeletal muscles showed (*a*) fibrous scars intimately woven with muscle to give an appearance of parcelling and sometimes of interruption of muscle fibres (Fig. 9A), (*b*) lymphorrhagia in the long axis of the muscle fibres (Fig. 9B), (*c*) oedema of the septa, and (*d*) haemorrhages.

In the oesophagus there was some thickening of the fibrous septa in the muscular layers. The thyroid showed marked thickening of the septa and some perivascular lymphocytic collections. The tonsil showed a small rim of lymphocytic tissue followed by grossly thickened and oedematous, almost hyalinized, fibrous tissue. This tissue contained markedly hyalinized small arteries, with an uninterrupted, somewhat thickened, internal elastic lamella.

The Diagnostic Significance of the Histological Changes in the Nail Cuticle

Case 2.—A widow, aged 55, two to three weeks before the onset of symptoms was stung by a bee on the left upper arm, which swelled up and was painful for two weeks. The main illness began six weeks before admission to hospital, when the fingers of both hands swelled up and became blue and painful on movement. There was also a dark brown swelling round the cuticles of the nails, and the pulps of the fingers were tender. Both wrists became swollen and painful at the same time. Three weeks later the ankles swelled and the thighs became painful anteriorly. A week later, pain, particularly on movement, spread to the shoulders and upper arms, but improved later. Some puffiness of the eyelids was observed on waking one week before admission, and a pleuritic pain was felt substernally and in the left axilla. The previous history contributed nothing, apart from attacks of "bronchial asthma."

She was admitted to the Central Middlesex Hospital on November 24, 1953. On examination she was pale and tired-looking, with angular stomatitis. The temperature was 100.5°. Blood pressure 190/120 mm. Hg. Respiratory movements were poor, and there was some dullness of percussion at both bases. The fundi showed bilateral soft exudates. No other abnormal neurological signs were found.

The cutaneous changes were brown crusts around the cuticles of all the nails of both hands. The small joints of the hands and the wrists were stiff, the left being worse than the right. Weakness led to difficulty in sitting up. Dermatomyositis was diagnosed clinically on account of weakness and the nail changes.

Investigations showed an E.S.R. of 40 mm. in one hour. Serum proteins were 5.5 g. per 100 ml. (albumin 3.2 g., globulin 2.3 g.). The 24-hour creatinine and creatine excretions were 830 mg. and 119 mg. respectively (within normal limits). The blood urea level was 28 mg. per 100 ml., and the serum potassium (two days before death) 4.5 mEq/l. (17.5 per 100 ml.).

A skin and muscle biopsy of the right deltoid on December 4 showed slightly flattened papillae with oedema of the dermis. Pieces of muscle showed no inflammatory reaction or vascular changes and no evidence of dermatomyositis.

Radiography of the chest showed patchy consolidation and collapse at the right base. Three blood cultures were sterile.

Despite the negative biopsy and normal creatine metabolism, A.C.T.H. therapy was begun on December 8 with 50 mg. A.C.T.H. b.d. intramuscularly, reduced later to 20 mg. b.d. Little change was noticeable, apart from a fall in temperature to normal and changes in the radiograph suggesting pulmonary oedema. On December 20 she became much worse, with cyanosis and dyspnoea, a temperature of 100° F., and signs of left basal pneumonia. She died on December 23, 10 weeks after the onset of symptoms.

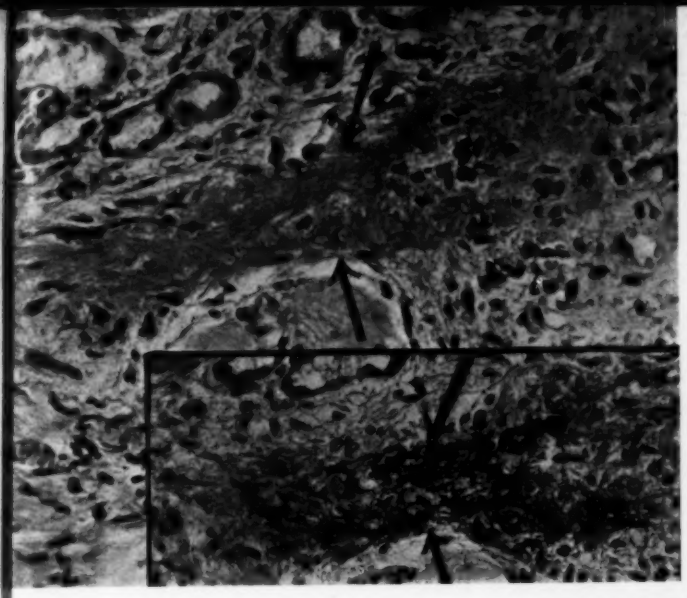


FIG. 8.—Case 1: kidney. Area of fibrinoid necrosis in the stroma (arrow) (haematoxylin and eosin, × 300). Inset: the same, stained for fibrin (P.T.A.H., × 300).

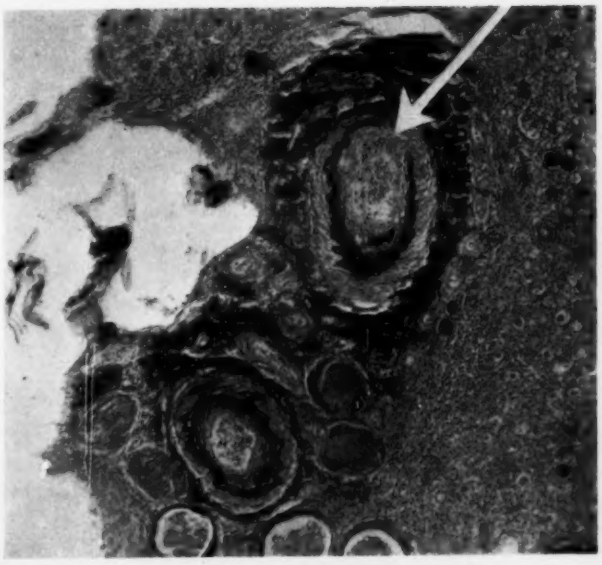


FIG. 7.—Case 1: kidney. Elastic van Gieson section, showing artery with reduplication of internal elastic. Another artery (arrow) shows a gap in the internal elastic membrane (× 80).

A

B

G. 9.—Case 1: skeletal muscle. A, Haematoxylin and eosin section showing fibrous scars (× 160). B, Haematoxylin and eosin section showing lymphorrhagia (× 300).

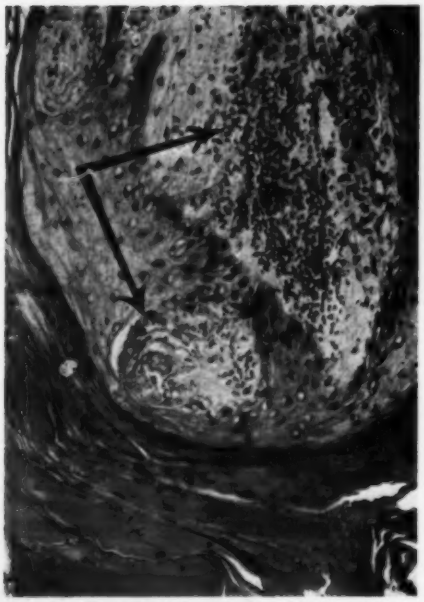


FIG. 10.—Case 2: histological section through nail bed showing intra-epidermal bulla filled with fibrin and capillary haemorrhage in the core (haematoxylin and eosin, × 160).

FIG. 12.—Case 2: nail bed. Section showing capillary thrombosis (arrows) (haematoxylin and eosin, × 300). Inset: the same, fibrin preparation (arrows) (× 300).

FIG. 11.—Case 2: nail bed. Section through intra-epidermal bulla, filled with fibrin and fibrinoid material (haematoxylin and eosin, × 300). Inset: the same. Fibrin preparation (P.T.A.H., × 300).

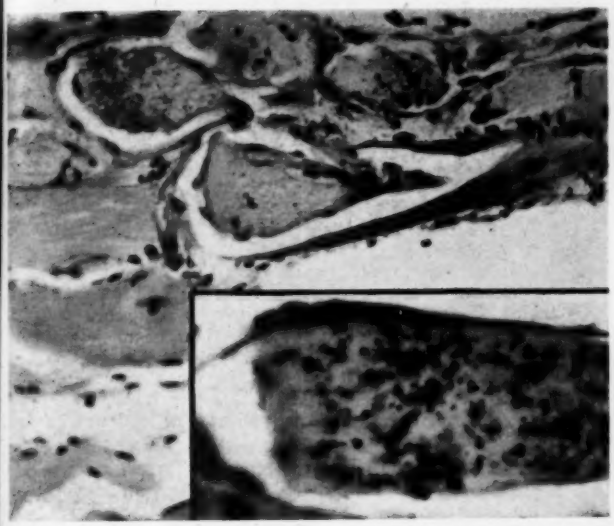
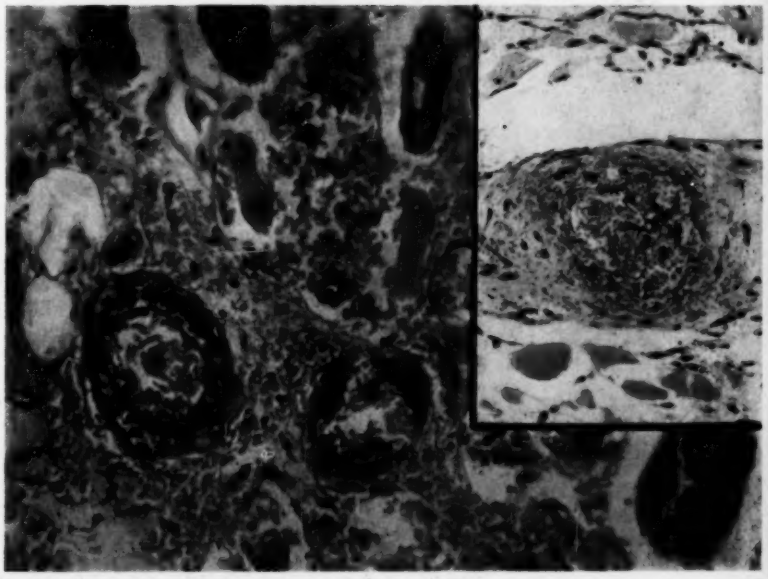


FIG. 13.—Case 3: peripheral muscle. Waxy degeneration with syncytia and calcification (haematoxylin and eosin, × 300). Inset: close-up of calcified degenerated muscle fibres (× 700).

FIG. 14.—Case 3: peripheral muscle. Venous thrombosis with haemorrhage. Fibrin stain (P.T.A.H., × 300). Inset: the same (haematoxylin and eosin, × 160).



Necropsy (No. 53/561).—The body was that of a middle-aged obese woman. There were no obvious skin changes apart from some roughening and swelling of the cuticles of the finger-nails. All lobes of the lungs were consolidated, with some right basal adhesions. The heart weighed 440 g. with considerable left ventricular hypertrophy but no gross lesions. The alimentary system appeared normal. The kidneys showed a normal pattern. The thyroid weighed 90 g., with a pale, fleshy cut surface. The skeletal muscles appeared grossly normal.

Histology.—The skin of the nail bed showed marked subpapillary congestion, capillary haemorrhages, and oedema (Fig. 10). In one area there were deposits of fibrin (verified by phosphotungstic acid haematoxylin staining) and fibrinoid material in the tips of three dermal papillae (Fig. 11). This material lay extravascularly and was distinct from fibrin in the dermal capillaries, which showed some thrombus more proximally (Fig. 12). There was some disorganization of the basal layer of the epidermis, which gave the impression of the formation of a small "bulla" containing fibrin at the dermo-epidermal junction (Fig. 10).

In the skin of the arm the papillary body was flattened with subpapillary oedema.

There was congestion of the lung with chronic atelectasis and carnification with occasional squamous metaplasia. Collagen, but no elastic fibres, was present in the carnified areas (Weigert-Van-Gieson stain).

In the kidney a fair number of glomeruli showed fibrinoid necrosis as well as hyalinization of afferent arterioles. There was some reduplication of arteriolar internal elastic laminae.

Oedema and central cortical congestion were seen in the suprarenal, with areas of fine fat droplets in the zona fasciculata.

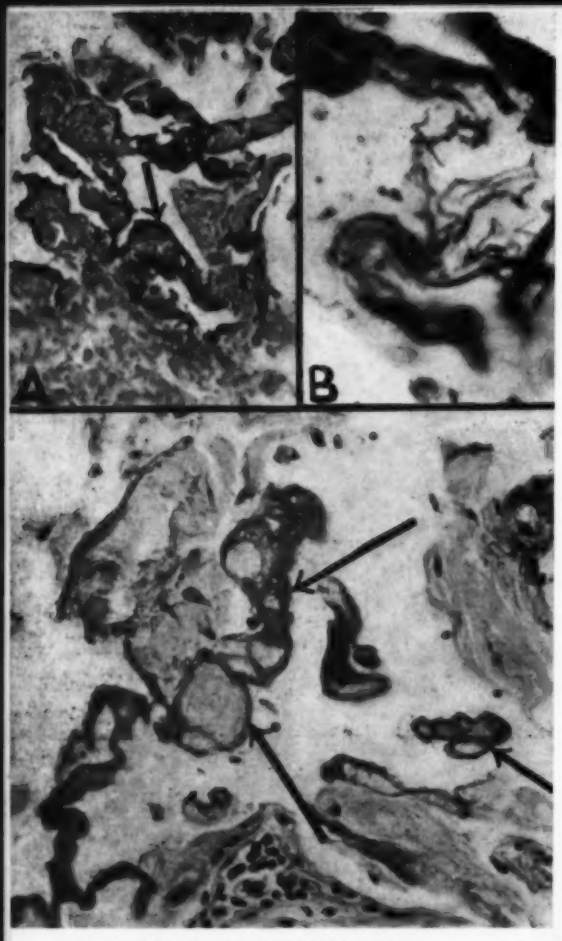


FIG. 15.—Case 3: eyelid. Histological section. Cap-like deposition of fibrin on oedematous collagen (arrows) (haematoxylin and eosin, × 300). Inset left: the same, fibrin stain P.T.A.H., × 160). Inset right: combined van Gieson and Wilder stain for argentaffine fibres (× 1,350). Fine argentaffine fibres preserved in oedematous areas without collagen affinity (stained yellow).

Peripheral fatty infiltration was found in the liver.

Sections from several areas of the brain and from the pituitary, thyroid, pancreas, ovary, myocardium, tongue, peripheral muscle, and ileum showed no appreciable changes.

Suprarenal Changes

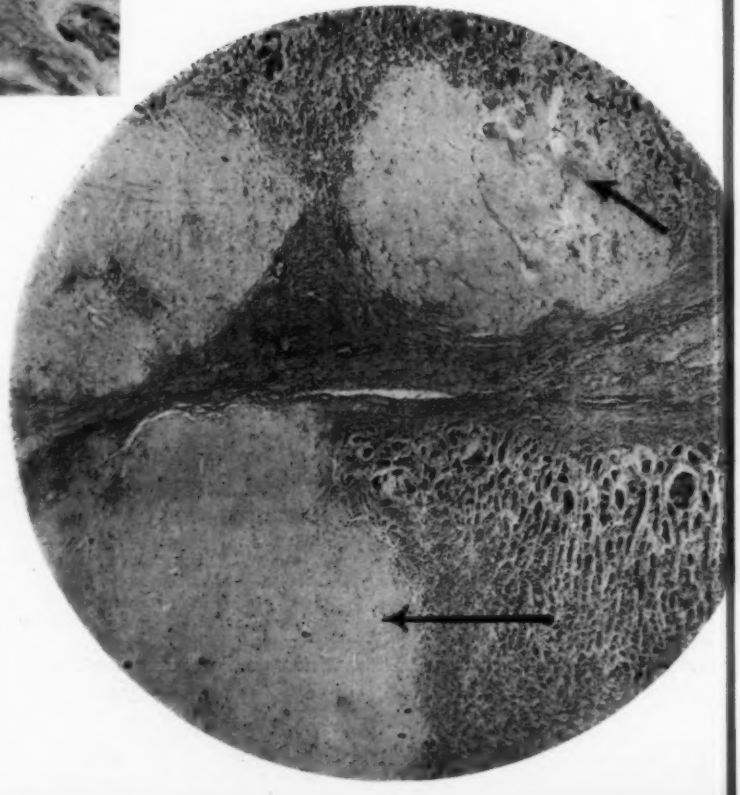
Case 3.—A man aged 69 was admitted on May 31, 1952, to another hospital complaining of pains in the upper and lower limbs. He had also had angina pectoris for six years. On examination there was oedema of the lower parts of both legs, splinter haemorrhages under the nails of several fingers, a soft systolic apical murmur, and ulcers over both heels, with small red infiltrated areas, up to 0.5 cm. in diameter, on the sole of one foot and the tips of two toes. There was remittent pyrexia. Oedema developed in one arm, the lower back and thighs, accompanied by weakness of the legs and trunk. The knee jerks became unobtainable after three weeks. The E.S.R. was 25 mm. in one hour. Blood platelets were normal in number.

A biopsy of the vastus lateralis (N.H. 9/52) sent for opinion to us showed waxy degeneration with loss of nuclei and striation, and fine, droplet-like calcification (Fig. 13); syncytia with more or less muscular degeneration and collections of nuclei in the sarcolemma (sarcolytes); lymphorrhagia in the perivascular septa, eosinophils in the stroma, and oedema of the perimysium. There were no appreciable vascular changes. The patient died on June 22.

Necropsy.—Post-mortem examination showed pallor of many skeletal muscles. There was an ulcer in the second part of the duodenum, 1.5 cm. in diameter, and some coronary atheroma. Pieces of tissue were sent to us for histological examination.

Histology.—In addition to the changes seen in the biopsy material, the vastus lateralis showed an area

FIG. 16.—Case 3: suprarenal. Fibrous plaques (arrows) (haematoxylin and eosin, × 35).



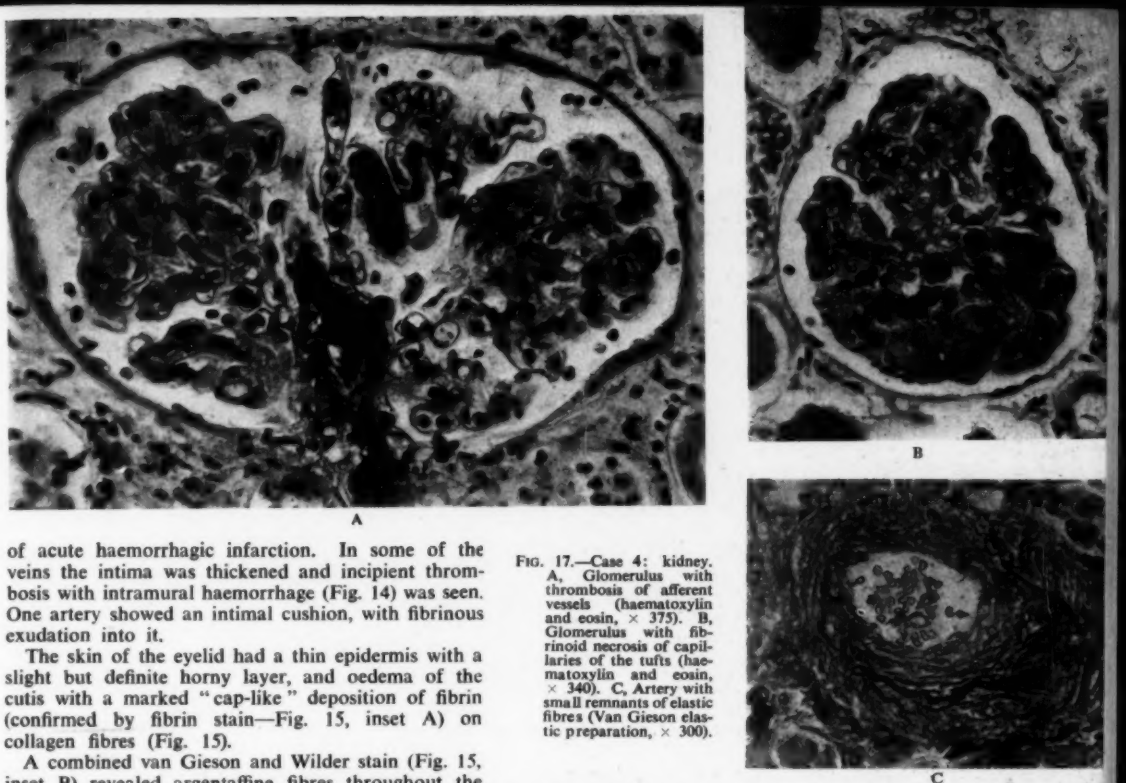


FIG. 17.—Case 4: kidney. A, Glomerulus with thrombosis of afferent vessels (haematoxylin and eosin, × 375). B, Glomerulus with fibrinoid necrosis of capillaries of the tufts (haematoxylin and eosin, × 340). C, Artery with small remnants of elastic fibres (Van Gieson elastic preparation, × 300).

of acute haemorrhagic infarction. In some of the veins the intima was thickened and incipient thrombosis with intramural haemorrhage (Fig. 14) was seen. One artery showed an intimal cushion, with fibrinous exudation into it.

The skin of the eyelid had a thin epidermis with a slight but definite horny layer, and oedema of the cutis with a marked "cap-like" deposition of fibrin (confirmed by fibrin stain—Fig. 15, inset A) on collagen fibres (Fig. 15).

A combined van Gieson and Wilder stain (Fig. 15, inset B) revealed argentaffine fibres throughout the yellow-staining, fibrinoid ground substance of the "caps." The "caps" are periodic acid positive.

The skin of the toe had a thin epidermis with a thick, horny layer, conspicuous oedema of the cutis, and occasional deposition of fibrin.

The suprarenals showed large, well-defined fibrous plaques up to 0.4 cm. in diameter in the stained section, replacing most of the parenchyma (Fig. 16). They did not contain amyloid, and were of collagen, fairly well vascularized. The capsule was thickened, oedematous, and hyaline. There were no appreciable vascular changes.

The tongue had a rough surface with sub-epidermal lymphocytic collections. The muscle showed slight, but definite, degenerative changes.

The aorta showed atheroma and round-celled collections round the vasa vasorum. The adventitial vessels showed concentric intimal thickening.

The liver showed some thickening of the peripheral portal septa.

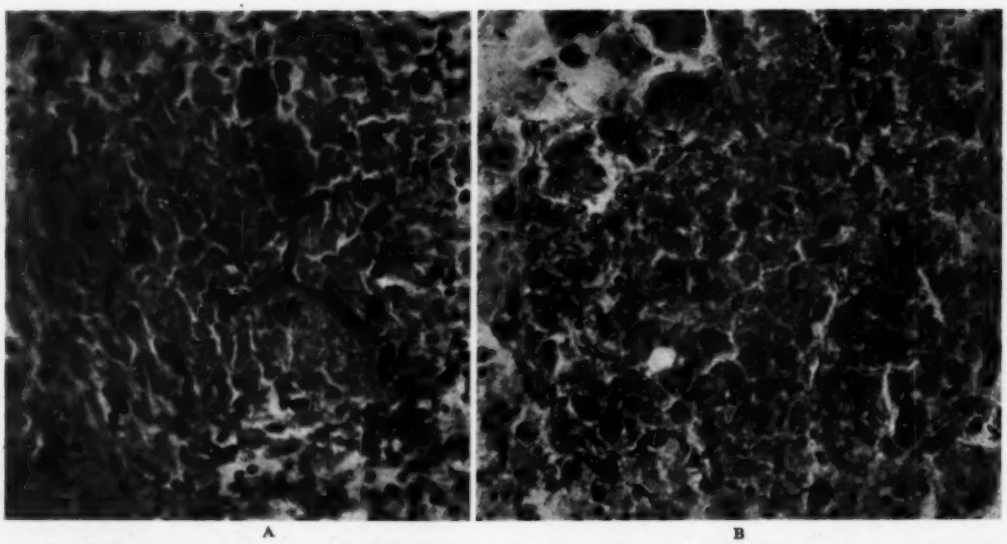
Progressive Diffuse Sclerosis with Focal Fibrinoid Necrosis in the Suprarenals

Case 4.—A woman aged 50 was admitted complaining of blueness, coldness, and pain in the feet and hands, progressing for the past 20 years. For six years she had suffered from arthritis. Examination showed an emaciated woman, with atrophic, shiny skin over the whole body, erythematous blotches on the face, and cyanosis of fingers and toes, with incipient gangrene and ulceration of the fingers. She also had signs of congestive heart failure, in which she died.

Necropsy (46/270).—The tonsils were dark red, ulcerated, and necrotic. There were some light pleural adhesions. The lungs had the consistency of consolidation in the lower lobe, but the cut surface showed no actual consolidation, only many haemorrhagic areas up to 2 × 2 cm. in diameter. The appearances resembled those of "rheumatic" lung. The pulmonary arteries appeared normal. The heart showed mitral stenosis with mural thrombus in the apical portion of the interventricular septum. There was also a haemorrhagic area in the posterior part of the septum, 3 × 1 cm. in diameter. The lower parts of the oesophagus and cardia were almost black and oedematous. The kidneys showed a regular granulation, with many sub- and intra-capsular haemorrhages. The cut surface showed a variegated picture of small haemorrhages and yellow flecks.

Histology.—The papillae of the skin of the chest wall were flattened, with marked oedema of the cutis beneath.

In the kidney there were some small cortical infarcts with central haemorrhages and intra- and extra-tubular collections of lymphocytes. The intima of medium-sized arteries was thickened concentrically. Some glomeruli showed thrombosis of afferent vessels and others fibrinoid necrosis of capillaries of the tuft (Fig. 17A and B), the latter change being rarer. Elastic stains showed that in some arterioles



A B

FIG. 18.—A, Tonsil. Focus of fibrinoid necrosis (haematoxylin and eosin, × 360). B, Suprarenal. Similar focus of fibrinoid necrosis (×300).

there were gaps in the elastic laminae similar to those seen in the kidney of Case 1 (Fig. 17C).

The tonsils showed sub-epithelial areas of fibrinoid necrosis around congested capillaries, in which the blood appeared to be static (Fig. 18A).

In the suprarenal there were ill-defined foci of fibrinoid necrosis scattered through the cortex (Fig. 18B). A few lymphorrhages were present.

In the tongue there was an oedematous conglutination of the connective tissue between the papillae and the muscle with lymphocytes. In addition, haemorrhages were seen round post-capillary veins. The oesophagus showed a picture similar to that in the tongue. The myocardium showed sub-endocardial haemorrhagic infarction. In the lungs there was intra-alveolar oedema and an exudate of lymphocytes into the bronchi. The liver showed severe passive congestion and the spleen marked oedema.

Transitional Renal and Muscular Changes

Case 5.—A man aged 46 was well until the onset of rheumatoid arthritis in the feet, shoulders, and knees in 1950. Butazolidin, begun in October, 1953, relieved most of his symptoms. He developed clinical signs of lesions of the fifth, ninth, and tenth cranial nerves on the right side in January, 1954. The spleen became palpable. Investigations revealed a blood pressure of 190/100 mm. Hg with papilloedema and retinal exudates. The urine contained protein. The globulin content of the blood was 6.12 g. per 100 ml., albumin 2.88 g. per 100 ml., zinc sulphate turbidity 42 units per 100 ml., and a blood urea level (terminally) of 240 mg. per 100 ml. (One month before the blood urea had been 32 mg. per 100 ml.) The blood showed a leucocytosis of 18,000 per. c.mm. with 67% neutrophils. The maximum erythrocyte sedimentation rate was 105 mm. in one hour. Bone marrow biopsy showed one doubtful lupus erythematosus cell. The clinical diagnosis was considered to lie between acute disseminated lupus erythematosus and polyarteritis nodosa. The patient died in uraemia within two weeks of admission.

Necropsy (54/204).—The body was that of a well-nourished man. The brain appeared normal externally. The lungs showed some grey hepatization, and there was some fibrinous pleurisy. The heart showed hypertrophy of the left ventricular wall. The caecum showed a small mucosal slough. The kidneys weighed 215 (right) and 275 (left) g. respectively. The capsules stripped easily, leaving smooth surfaces showing occasional petechiae and some yellowish flecks. The cut surface was of brownish red, and the corticomedullary pattern was almost completely lost, without focal infiltrations or haemorrhages and without arterial prominence. The pancreas weighed 160 g. and had a firm nodular consistency. Two areas of necrosis were present, the larger, measuring 3 × 2 × 0.5 cm., being in the head; it was sharply defined, with a yellowish homogeneous surface and an adjacent area of haemorrhagic necrosis which seemed grossly to contain a thrombosed artery. The smaller infarct measured 1.5 × 1 × 1 cm. and was situated at the junction of the left and middle thirds; it showed a similar cut surface, without haemorrhage. There was no adjacent fat necrosis. The skeletal muscles and other major viscera showed no gross abnormalities.

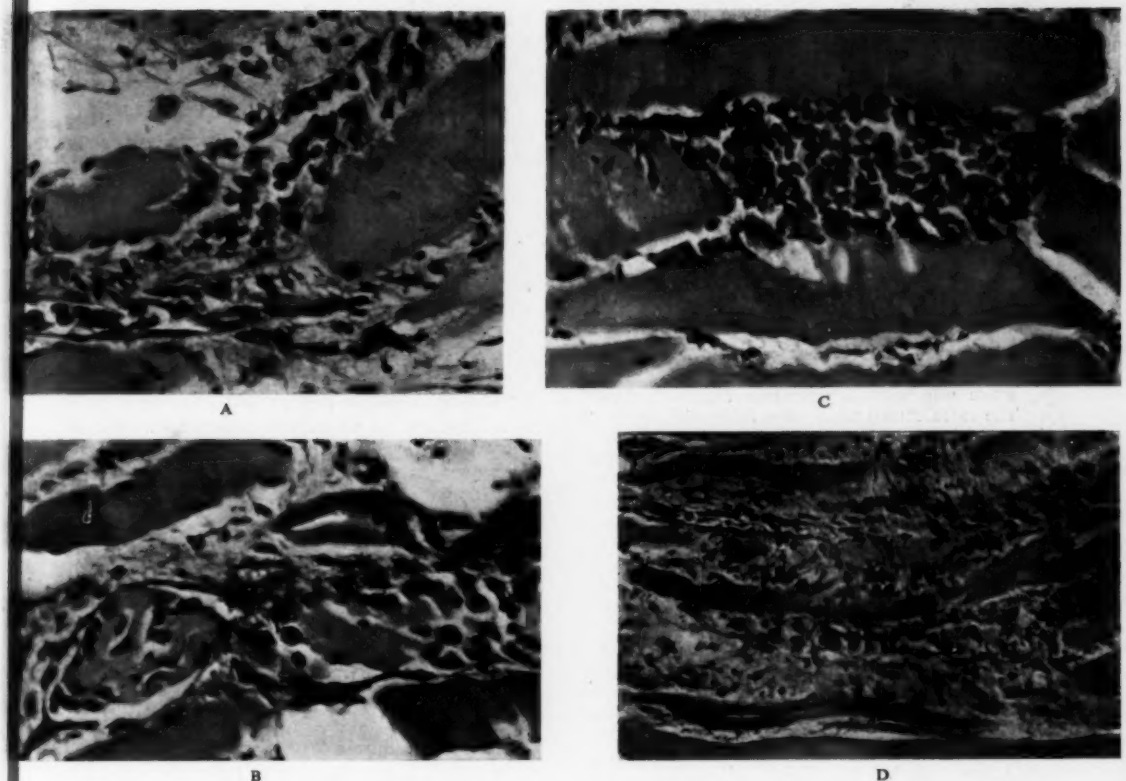


FIG. 19.—Case 6: peripheral muscle. A, Waxy degeneration of muscle fibres with sarcolemmal cell reaction (haematoxylin and eosin, × 300). B. The same. Sarcolemmal cells occupy deep lacunae in the dissolving musole bundles (haematoxylin and eosin, × 300). C, Part of sarcolemmal tube filled by sarcolemmal cells mixed with some polymorphs (haematoxylin and eosin, × 300). D, The same. Thickening and oedema of stroma (haematoxylin and eosin, × 160).

Histology.—In the glomeruli the most striking change was a focal fibrinoid necrosis of the tufts, with occasional "wire-loop" formations due to picking out of the basement membranes. Proliferative glomerulitis and capsulitis were not seen. There were a few hyalinized glomeruli. The tubules showed patchy necrosis, often severe and hyaline in appearance, occasionally with a collection of neutrophils. The lumina contained casts which were often brightly eosinophilic and positive for fibrin (P.T.A.H. staining). The vessels showed various lesions, the most conspicuous change being a fibrinoid necrosis of afferent glomerular and larger arterioles, with which a thrombosis was sometimes associated. In the periphery extensive hyaline and thrombotic lesions were often associated with subcapsular acute infarcts. The larger arteries showed hyperplastic intimal and medial sclerosis. There was moderate interstitial oedema with occasional lymphocytic collections. Staining for amyloid was negative.

Large areas of recent infarction, with peripheral neutrophil reaction, were seen in the pancreas. There were areas of haemorrhage with necrosed vessels, one of which contained thrombus. Arteritis was not seen. Many vessels showed hypertensive changes.

Some of the sections of peripheral muscle showed degeneration and fragmentation of muscle fibres, with cellular ingestion of fragments in the sarcolemmal tubes. There was a general increase of sarcolemmal nuclei and also some focal lymphorrhages with a few plasma cells.

Fibrinous pleurisy, with an organizing oedema and incipient bronchopneumonia in the parenchyma, was found. Many siderophores were present in the alveoli.

The caecum showed an infarcted mucosa associated with thrombosed vessels. Serosal vessels showed very severe intimal sclerosis.

Sections of the bone marrow, liver, spleen, thyroid, small intestine, suprarenal gland, and retina showed

no significant changes apart from those of hypertension. The Gasserian ganglia showed focal demyelination.

Dermatomyositic Changes Resembling Experimental Findings

Case 6.—A woman aged 37 was admitted to another hospital with an obscure pyrexia, thought to be due to pneumonia. Soon after admission solid subcutaneous oedema of the arms and thighs was noted. A clinical diagnosis of dermatomyositis was made and cortisone was given, with rapid improvement. A biopsy of the skin and biceps was sent to us for opinion.

Histology.—The sections showed a variety of characteristic changes.

There was waxy degeneration of muscle bundles.

The sarcolemmal tubes were filled with fairly large polygonal cells with somewhat hyperchromatic, round, elliptic, or spindly nuclei. These cells had engulfed or clustered round fragments of necrotic muscle (Fig. 19A). Sometimes they seemed to lie in deep lacunae or else form round muscle bundles (Fig. 19B). Besides these there were peripheral remnants of muscle tissue adherent to the sarcolemmal wall, leaving a central space (" pseudo-vessels ").

Syncytia, mostly forming seams along degenerating muscle fibres, were found.

Polymorphs, including a few eosinophils, formed micro-abscesses replacing parts of muscle bundles within the confines of the sarcolemma (Fig. 19C).

The stroma, which contained a few plasma cells, some capillary haemorrhages, a few polymorphs, and somewhat juicy fibroblasts (Fig. 19D), was thickened and oedematous.

The papillary body, with marked oedema of the cutis, was flattened.

CLINICAL AND HISTOLOGICAL CONCLUSIONS

Diagnosis

Looking back at the descriptions of these cases, the first point to be discussed is diagnosis.

In Case 1 the patient, developing dermatomyositis 11 years before death, died of a disease which accords with the descriptions of generalized scleroderma or progressive diffuse sclerosis.

In Case 2 the diagnosis was doubtful. The clinical picture could equally well have fitted a case of acute disseminated lupus erythematosus, a rash often being absent in this condition. Clinically the parallel is close ; in both disseminated erythematosus and dermatomyositis there is fever, muscular weakness, oedema of the eyelids, and lesions around the cuticles of the nails. Pleurisy is perhaps more in favour of lupus erythematosus, while the absence of anaemia and leucopenia and the normal serum proteins are against it. It is unfortunate that lupus erythematosus cells were not searched for.

In Cases 3 and 6 the clinical diagnosis was consistent with subacute dermatomyositis, which was confirmed by biopsy.

Case 4 was clinically one of scleroderma.

In Case 5 the clinical diagnosis of polyarteritis nodosa was not borne out by the histological findings. These included " wire loop " glomeruli and capillary and arteriolar thromboses in the kidney, changes suggestive of disseminated lupus erythematosus. It should be added that a doubtful lupus erythematosus cell was found in the bone marrow, but lupus erythematosus cells have been observed by Cohen (1954) in rheumatoid arthritis and can therefore not be considered diagnostic. Finally, the striking reversal of the albumin : globulin ratio, such as in this case, is not normally seen in polyarteritis nodosa, and supports the diagnosis of disseminated lupus erythematosus.

Histology of the Affected Organs

Lungs.—These were affected principally in Case 1. According to Ellman and Cudkowicz (1954) and Evans and Parker (1954) the pulmonary changes recorded in scleroderma are diffuse fibrosis, fine nodulation, cyst formation, and pleural fibrosis. Getzowa (1945), who gives a detailed description of these changes, shows a photograph of the cystic lesions, resembling a " honeycomb " lung. Other authors lay stress on the diffuse fibrosis without macroscopic changes (Weiss, Stead, Warren, and Bailey, 1943). The appearance of the right lung in the present case differs considerably from those already described—a picture of thick, fibrous bands with large fibrous plaques obliterating most of the parenchyma. Histologically, the striking feature is the presence of much elastic tissue in the fibrous areas, in contrast to Getzowa's cases, in which elastic fibres had disappeared from the fibrous areas, indicating a probable sequence of collapse followed by fibrosis. The small cysts are produced by a compensatory emphysema. The fibrinoid lining of the alveoli was described by Getzowa and resembles that seen sometimes in the so-called rheumatic lung (Hadfield, 1938). It has been pointed out since that these membranes are not specific of rheumatic aetiology. We observed several classical examples in cases with acute terminal circulatory failure, e.g., after thoracoplasty. Nevertheless, the frequency of such changes in acute rheumatic diseases, especially when combining with organizing changes (" Masson's

bodies "), remains impressive (Neubuerger, Geever, and Rutledge, 1944).

Kidney.—The main question in respect of this organ is the incidence and specificity of arteriolar fibrinoid necrosis in scleroderma, as described by Moore and Sheehan (1952), of "wire loop" glomeruli in disseminated lupus erythematosus, as described by Klemperer, Pollack, and Baehr (1941), and of gaps in the elastic lamellae as seen in burnt-out polyarteritis nodosa (Pagel, 1951).

The "scleroderma" change was absent in Case 1, present in Cases 2 and 4. Whereas Case 1, in spite of its absence, was a typical example of diffuse scleroderma ("visceral sclerosis"), Case 2, in which it was present, was not. The "L.E.D." change was found in Case 5 only, in which it established the diagnosis of disseminated lupus erythematosus, against the clinical diagnosis of polyarteritis nodosa, as it can for practical purposes be considered characteristic of disseminated lupus erythematosus (Allen, 1951).

The "polyarteritis nodosa" change occurred in Cases 1 and 4, which were both of scleroderma.

Two other less characteristic vascular changes were capillary and arteriolar thromboses, as seen in Case 5.

Of non-vascular kidney changes, the area of fibrinoid necrosis in the stroma is of interest, for such extravascular renal foci do not seem to have been observed previously in scleroderma, although they are well recognized in the skin (see Case 3).

Liver and Pancreas.—The liver was grossly changed in Case 1 only (a case of scleroderma). Beerman (1948) remarks that little has appeared in the recent literature on hepatic involvement in scleroderma. This remark still holds good. Brock (1934) noted in his Case 4 an increase of periportal connective tissue. Perdomo de Fernández, Ravera, and Piovano (1951) performed a liver biopsy on a man aged 43 with scleroderma, whose liver was palpable two fingerbreadths below the costal margin. He comments that few cases of scleroderma have hepatic symptoms and that the abdomen is often difficult to palpate because of the rigidity of the wall. Histological examination showed foci of sclerosis consisting of whorls of collagen fibres showing some hyaline degeneration. There were also some collections of round cells and fibroblasts. Both lesions were accompanied by discrete hepatocellular degenerative lesions—fatty changes, with bile pigment deposits. Harvier and Bonduelle's case (1947), a woman aged 43, showed radiologically calcification of the hepatic capsule. Biopsy was not performed. Goetz describes the "deposition of a peculiar amorphous material in a cirrhotic liver of an unusual type," but gives no further details of these cases. Beigelman *et al.* (1953) describe centrilobular hepatic fibrosis in their Case 9. The findings in the present case seem to correspond fairly closely with those of Perdomo de Fernández *et al.*, the portal changes being more advanced with little parenchymal damage. In Harvier and Bonduelle's case there was probably a similar capsular fibrosis, in which calcium deposits had been laid down. It seems then that the tendency to fibrosis extends to the portal tract and capsule of the liver in some cases.

The pancreas was involved in Case 5, in which there was evidence of malignant hypertension. Pancreatic necrosis due to arterial thrombosis in malignant hypertension has been reported (Pagel and Woolf, 1948) and we have also seen recanalized thrombosis without necrosis of the pancreas in a case of malignant hypertension. The lesion in the caecum in the present case was probably of a similar nature.

Skin.—Lesions of scleroderma were found in Case 1 (scleroderma), in Case 2 (a transitional case between dermatomyositis and disseminated lupus erythematosus), in Case 3 (dermatomyositis), and in Case 4 (dermatomyositis).

Nail bed changes were seen in Case 2 (transitional between dermatomyositis and disseminated lupus erythematosus).

Lesions of the nail cuticles in dermatomyositis are well recognized (Keil, 1942) and may be an early symptom. Searching through the cases referred to by Keil and through subsequent literature, we have been unable to find any account of the histological appearance of these lesions and so are unable to compare our own findings with any

FIG. 20.—Case 2A: naked-eye appearances of nail bed changes in lupus erythematosus disseminatus. Splinter haemorrhages in nails, in addition to multiple petechiae, swelling, and blistering of nail cuticulae.

FIG. 21.—Case 2A: fibrin thrombi (arrows) in capillaries of the cutis. Thinning of epidermis with epidermolysis (arrows). (Haematoxylin and eosin, × 160.)

others. The cases of Pick (1935) and Engelhardt (1935) seem to correspond clinically to our own, but no histological report was given. The picture we found does, however, demonstrate that ubiquitous hall-mark of the "collagen" diseases, fibrinoid necrosis. The "bulla" seems to be the result of epidermolysis. Liquefactive degeneration of the basal epidermal cells is also a feature of lupus erythematosus (Klemperer *et al.*, 1941; Lever, 1949). The changes in this case have a particular point of interest because they show a combination of capillary thrombosis with extravascular fibrinoid necrosis. Such a combination is not normally seen. Usually we have either the picture of acronecrosis with capillary thrombosis, as seen in thrombocytopenic purpura (Moschcowitz, 1925; Baehr, Klemperer, and Schifrin, 1936) or in subacute bacterial endocarditis (Pagel, 1949) and thrombotic micro-angiopathy (Symmers, 1952), or else that of extravascular fibrinoid necrosis as seen in collagen disease (disseminated lupus erythematosus, dermatomyositis). In polyarteritis nodosa, the classical collagen disease of blood vessels, no evidence of primary thrombosis can be found and sub-endothelial hyaline deposition, said to precede acrothrombosis (Gore, 1950), differs from the early polyarteritic changes (Pagel, 1951).

Thrombosis observed in the same area is not incidental, however. This is shown by its occurrence in a similar setting in the skin changes of disseminated lupus erythematosus. As an example the case may be briefly described of a man aged 62 years (Case 2A), who died after a five months' illness suggestive of disseminated lupus erythematosus — erythematous rash, joint pains, and progressive loss of weight. No lupus erythematosus cells were found in the bone marrow and a skin biopsy was not helpful. Terminally the rash became bullous and there were swelling, cyanosis, and epidermolysis of the nail folds (Fig. 20). There was no response to treatment with A.C.T.H. and cortisone. The post-mortem examination (P.M. 54/265) showed grossly only a basal bronchopneumonia and a diffluent splenic pulp. Significant histological changes were restricted to the skin and nail fold, and were similar at both sites. The epidermis was flattened and atrophic, more so in the skin than in the nail fold, with liquefaction degeneration of the basal layer (Fig. 21). The corium was markedly oedematous with capillary congestion. Some of the dilated capillaries contained fibrin thrombi (Fig. 21). Small haemorrhages had occurred in some of the papillae. In addition, there was fibrinoid degeneration in the deeper corium. There was a moderate lympho-

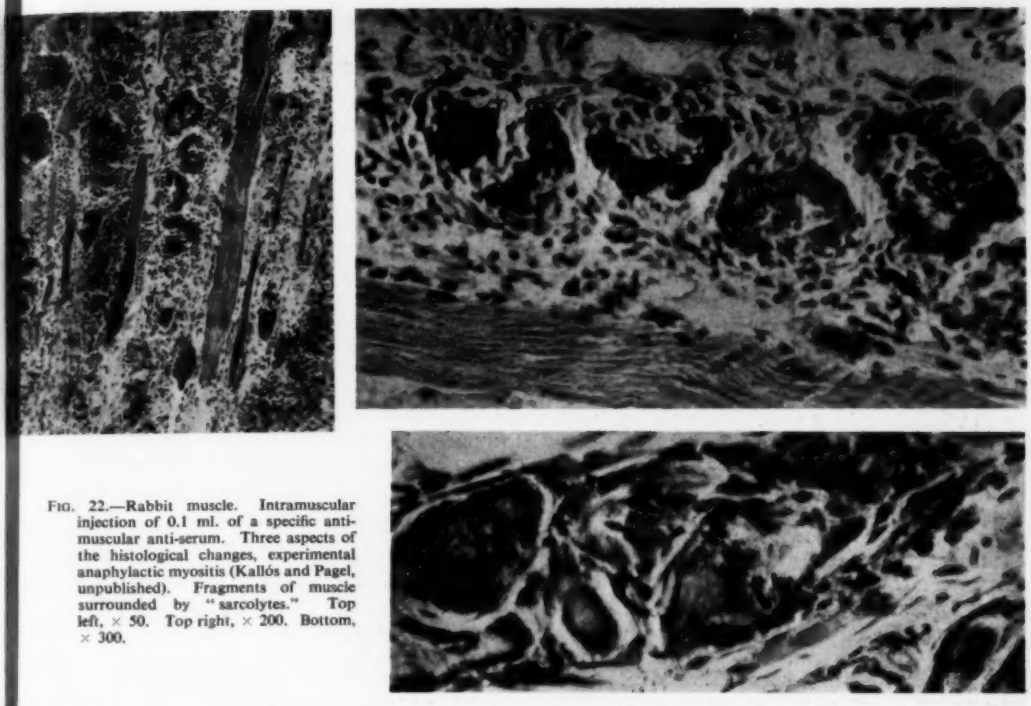


FIG. 22.—Rabbit muscle. Intramuscular injection of 0.1 ml. of a specific anti-muscular anti-serum. Three aspects of the histological changes, experimental anaphylactic myositis (Kallós and Pagel, unpublished). Fragments of muscle surrounded by "sarcolytes." Top left, × 50. Top right, × 200. Bottom, × 300.

cytic infiltration of the corium, sometimes localized round the appendages.

These changes are fairly characteristic of acute lupus erythematosus, in which we would like to include capillary thrombosis, although Klemperer *et al.* (1941) on the basis of a much more extensive material considered thrombosis to be uncommon in this disease. We regard the thrombosis to be independent of vascular damage, which was absent in the walls of thrombosed vessels.

It should be mentioned, however, that it is the combination of capillary thrombosis and fibrinoid necrosis rather than their occurrence singly which seems to be characteristic. For, in one of three control nail beds selected from random post-mortem material (Nos. 54/182, 54/183, 54/184), a case of coronary thrombosis in a male aged 74, a small fresh, fibrin thrombus was seen in an intra-papillary vessel, whereas no appreciable changes were seen in the two other control cases.

In connexion with the acrothrombosis and capillary haemorrhages seen in the present case, it should be noted that, according to Keil, gross haemorrhages may occur in the nail beds in disseminated lupus erythematosus, and splinter haemorrhages in the nail beds are a feature of dermatomyositis.

A feature worthy of comment is the fibrinoid "caps" lining bundles of collagen fibres in the eyelid. A combined van Gieson-Wilder stain shows that the "caps" consist not of fibrin alone but of a non-collagen ground substance in which argentaffine fibres are present. It is tempting to assume that this ground substance was originally collagen in a state of fibrinoid degeneration, and to compare the presence of argentaffine fibres with that typically observed in rheumatic and para-rheumatic conditions (Klinge, 1933; Pagel, 1951).

Muscle.—Muscle changes were absent in Cases 2 (transitional case) and 4 (scleroderma).

Of the others, Case 5 (disseminated lupus erythematosus) showed myositic changes which will be discussed in the final comment. In connexion with this case, it should be remembered that lesions of striped muscle are not uncommon in rheumatoid arthritis (Cruickshank, 1952).

The classical myositic changes were seen in Cases 1, 3, and 6. The changes in Case 6 indicate acute episodes superimposed on a more protracted,

though extensive, destruction of muscle. The acute changes are shown by micro-abscesses replacing parts of necrotic muscle bundles and by the perimysial haemorrhages. Chronic changes are shown by extensive collections of cells engulfing small fragments of necrotic muscle within the confines of the sarcolemmal tubes. The first impression is of inflammatory cells in vessels, but a closer study soon shows that the spaces are really sarcolemmal tubes, partly empty, partly filled with special cells, namely the sarcolytes described by Glücksmann (1934) in the metamorphosis of amphibia. These cells should be regarded as the associates, or perhaps the precursors, of the syncytia, the well-known products of muscular regeneration. Apart from the polymorphs, the inflammatory cells, notably plasma cells and macrophages, are rare though present here and there in the stroma. These pictures invite a comparison with the experimental findings by Kallós and Pagel (1937) produced by injecting a muscular antiserum into rabbit muscle. Here, too, fragments of muscle, often within the sarcolemma tubes, were found. These formed, together with distinctly eosinophilic sarcolytes, small granulomata and syncytia comparable to those seen in the present case (Fig. 22).

Suprarenals and Tonsils.—The extensive focal fibrosis of the suprarenal cortex seen in Case 3 seems to be a noteworthy feature. From the soft, oedematous nature of the fibrous tissue we would suggest that the change is related to the disease and is not accidental or congenital. In view of the suprarenal changes found in Case 4—foci of fibrinoid necrosis—it is not unlikely that the focal fibrosis represents the terminal stages of such necrotic foci. In Case 4 areas of focal fibrinoid necrosis were also found in the tonsil.

DISCUSSION

It can be seen from the discussion of the cases that the differential diagnosis of dermatomyositis, progressive diffuse sclerosis, and disseminated lupus erythematosus can be a matter of the utmost difficulty. Case 3 is the most straightforward and one can with fair confidence diagnose it as a subacute dermatomyositis, both clinically and histologically. With Case 1, however, we encounter difficulty. Are we to call it chronic dermatomyositis with visceral changes or progressive diffuse sclerosis supervening upon a former attack of dermatomyositis? The continuity of the disease since the original onset 11 years before death would favour the former diagnosis, but the visceral lesions were certainly those of a progressive diffuse sclerosis. Nor is histology helpful. Brock states that the late stages of the two diseases can be distinguished because in dermatomyositis "inflammatory infiltrations and a variety of degenerations of the parenchyma of muscles are outstanding features to the end." From his description, it seems he is referring to the round-celled infiltrations of muscle now known as "lymphorrhagia." They are also now known to be non-specific (Cruickshank, 1952; Russell, 1953). Freudenthal (1940) was able to demonstrate lymphorrhages in muscle in a case of scleroderma and concluded that "the histological changes in skin and muscle in generalized scleroderma and dermatomyositis show no essential difference." With this latter view we agree. We also feel that either chronic dermatomyositis has the visceral lesions of progressive diffuse sclerosis or that dermatomyositis merges imperceptibly into it. We therefore conclude that Case 1 is an instance of dermatomyositis terminating in progressive sclerosis.

Where then does Case 2 stand diagnostically? Clinically it appeared to be a case of dermatomyositis, but no muscular changes were found either during life or after death when the only relevant change was in the nail bed. Reports in the literature indicate the difficulties of other authors (Bradley, Drake, and Mack, 1951; Madden, 1950). Out of 21 biopsies in Madden's cases of disseminated lupus erythematosus, six showed nodular myositis. Fifty per cent. of them showed lupus erythematosus cells in marrow examination. He concluded that the "muscle changes in dermatomyositis and disseminated lupus erythematosus are not specific" and even discourages muscle biopsy as a major procedure for a very ill patient, recommending rather the examination for lupus erythematosus cells, despite the low incidence of positive results in the latter investigation. Histological evidence of the non-specific character of the muscular changes in dermatomyositis had already been adduced by Pagel *et al.* (1949). Degos, Garnier, Darnis, and Vissian (1949) described a case of subacute lupus erythematosus with dermatomyositis. Of Spühler and Morandi's (1949) two cases, the first showed diffuse scleroderma with Raynaud's syndrome, clinical dermatomyositis, and a Libman-Sacks endocarditis. They concluded that disseminated lupus erythematosus, dermatomyositis, and scleroderma are differing forms of reaction to a non-specific infection. In Clein's (1953) case of acute dermatomyositis the muscle biopsy (reported by us) was negative, but this was not considered to exclude dermatomyositis. The case bore a close resemblance clinically to

our Case 2, but showed no cuticular changes and had an erythematous rash. Retrospectively it is difficult to exclude also the possibility of an acute disseminated lupus erythematosus in Clein's case, which responded to cortisone. Later the patient developed a condition resembling rheumatoid arthritis. We suggest therefore that our Case 2 combines clinical features of dermatomyositis with histological features of progressive diffuse sclerosis and disseminated lupus erythematosus.

Case 5 illustrates a further aspect of the clinical diagnostic difficulty. Polyarteritis nodosa was considered here because the one possible lupus erythematosus cell seen was thought to be a "tart" cell rather than a true lupus erythematosus cell (the ingested nuclear fragment was sharply defined and retained a recognizable chromatin pattern) and was therefore disregarded. As the "tart" cell is supposed to be a precursor of the lupus erythematosus cell and as neither of these cells occurs in polyarteritis nodosa, this disregard seems to have been unjustified. The histological findings quite definitely excluded polyarteritis nodosa. Many of the changes found, necrotizing arteriolitis, capillary thrombosis, pancreatic necrosis, and myositis, are also found in other syndromes, and we are left with the distinctive "wire-loop" phenomenon. This case had features in common with progressive diffuse sclerosis, dermatomyositis, and disseminated lupus erythematosus.

Case 6 shows that dermatomyositis is primarily a degenerative and not an inflammatory lesion of muscle. The majority of the cells seen are young myogenic elements, probably sarcolemma cells, which engulf and cluster around fragments of necrotic muscle. They seem to be related to the syncytia seen in a similar site, at or lining, the sarcolemma and fragments of necrotic muscle. Inflammatory cells are secondary invaders; in the present case, polymorphs, including some eosinophils, form an addition somewhat unusual in dermatomyositis, whereas plasma cells, a conspicuous feature in some cases, are very scarce. Lymphocytes commonly seen in the form of "lymphorrhagia" are also scanty.

In none of these cases were the haematoxophil bodies said to be characteristic of disseminated lupus erythematosus (Klemperer, Gueft, Lee, Leuchtenberger, and Pollister, 1950) found. They were, however, present in a case previously reported by Pagel (1951, Fig. 7). The latter case showed a clinical picture of polyarteritis nodosa with consistently high eosinophilia, polyneuritis, and fleeting pulmonary infiltrations. An excised skin nodule showed extensive deposition of granules with intense affinity for haematoxylin—the picture of haematoxophil bodies. An additional feature was the distinct foreign-body reaction of tuberculoid type which they elicited. This observation of haematoxophil bodies in a case of polyarteritis nodosa confirms the findings of Worken and Pearson (1953) that these bodies need not be invariably associated with disseminated lupus erythematosus.

The distinctive features of the present series are summarized in Table I.

TABLE I

SUMMARY OF HISTOLOGICAL CHANGES IN PRESENT SERIES

Lesion	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Muscle changes resembling dermatomyositis	+	0	+	0	+	+
Muscle changes resembling experimental myositis ..	0	0	0	0	0	+
Skin changes resembling dermatomyositis ..	+	+	+	+	0	+
Skin changes resembling scleroderma	+	+	+	+	0	+
Visceral changes resembling progressive diffuse sclerosis ..	+	+	0	+	+	Not known
Visceral changes resembling disseminated lupus erythematosus ..	+	0	0	+	+	„

It would appear, then, from the increasing number of reports in the literature and from our own experience, that the clear-cut diagnosis of diseases of the collagen group (notably acute dermatomyositis and disseminated lupus erythematosus) is becoming progressively undermined, from both the clinical and histological points of view.

SUMMARY

The clinical and histological features of dermatomyositis, progressive diffuse sclerosis, and disseminated lupus erythematosus may overlap sufficiently to render differential diagnosis impossible in some cases. We suggest that such intermediate forms be grouped under the name of "viscero-cutaneous collagenosis."

From the material presented, the view that dermatomyositis and scleroderma are stages of the same disease is confirmed.

The changes in the nail fold, clinically regarded as characteristic of disseminated lupus erythematosus and dermatomyositis, are a clearly recognizable histological entity, consisting of patchy extravascular fibrinoid necrosis associated with capillary thrombosis—an unusual combination of collagen disease and acronecrosis.

One of the cases of dermatomyositis presented shows muscular changes strikingly similar to those reproduced experimentally by means of an anti-muscular serum.

Focal necrosis and focal fibrosis in the suprarenal are thought to represent the early and end stages of a process which can be associated with dermatomyositis.

We are indebted to Dr. Josephine Roth, Dr. Herbert Levy, and Dr. R. Aidin for material and opportunities for clinical pathological discussion, to Mr. L. G. Spain and Miss Marjorie Webb for technical assistance, and to Miss Yvonne Vincent for assistance with photography.

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INTERSTITIAL PLASMACELLULAR PNEUMONIA DUE TO *PNEUMOCYSTIS CARINII*

BY

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Interstitial plasmacellular pneumonia of young infants is a fairly common disease on the European continent. It has been described as a new morbid entity by Rössle (1923, 1928) and Loeschcke (1928) and in more detail by Benecke (1938), Ammich (1938), Feyrter (1927, 1941), Roulet (1941), Gloggengiesser (1951), and others. Nitschke (1940) published a thorough review of clinical symptoms and radiological findings, and Weisse (1951) recently wrote an extensive monograph. The disease occurs mainly, but not exclusively, in premature infants between 2 and 4 months of age, and almost exclusively in those who have been in hospital for some time. Clinically it is characterized by severe respiratory distress and cyanosis with little or no fever and no definite physical signs over the chest but marked radiological changes. Almost all reports are from Switzerland, Germany, Austria, and Czechoslovakia, only a very few from northern European countries (Linell, 1949 ; Gormsen, 1950), none from southern Europe, and none from outside the European continent.

The purpose of the present paper is to report the first case outside the European continent, to describe the histochemical characteristics of the peculiar honeycombed masses which fill the air spaces, to demonstrate the histopathological differences between plasmacellular interstitial pneumonia and other forms of interstitial pneumonia of infancy, to confirm the finding of Vaněk and Jírovec (1952) of the protozoon *Pneumocystis carinii* in the air spaces, and to discuss briefly the curious association with cytomegalic disease of salivary glands.

Case Report

The patient, a girl, was born at full term in an institution in the Midlands with a birth weight of 6 lb. She was 12 weeks old at the time of death. The mother suffered each winter from respiratory tract infection. The baby was well until the end of December, 1950, eight days before death, when she developed a nasal discharge. Four days before death she developed a cough which was occasionally paroxysmal and associated with vomiting. Next day tachypnoea and cyanosis were noticed. On admission, two days before death, she was distressed and cyanosed. Rhonchi and rales were heard over both lungs. Her temperature was 100° F., the white cell count 13,200 with 51% polymorphonuclear leucocytes. The mother had her usual respiratory tract infection in December. There was no history of respiratory tract infections in the maternity home where the baby was born.

Pathology

At the necropsy the pleurae were normal ; the trachea and the main bronchi contained a small amount of mucopurulent exudate but their mucosa appeared normal. The lungs were bulky, but there was no pus in the smaller bronchi and bronchioli. Scattered throughout all lobes of both lungs were ill-defined, greyish-red areas of consolidation, 2–3 mm. in diameter, and between these there were grey spots and broad grey strands, apparently corresponding to broadened interstitial tissue. The tracheo-bronchial lymph nodes were of normal appearance. The spleen was enlarged (23 g.). The Malpighian corpuscles were rather conspicuous, the pulp greyish-red and not diffluent. The liver showed moderate fatty changes, the kidneys cloudy swelling, the other organs no abnormality.

Microscopical Examination.—In the lungs all alveolar ducts were distended and, like the less distended alveoli, filled with a peculiar foamy or honeycombed material (Fig. 1).

In some of the air spaces there were also red blood cells which showed little alteration. Alveolar macrophages were seen only in a few alveolar ducts and alveoli. Alveoli, alveolar ducts, and respiratory bronchioles were all lined with epithelial cells which were usually cuboid, sometimes low columnar, and sometimes moderately flattened (Fig. 2). These cells were rounded at the free borders. They had large vesicular nuclei with a single nucleolus and a small amount of cytoplasm

which was finely granular. The granules stained pale blue with haematoxylin and eosin, dark purple with Giemsa. Because of this modified lining of the air spaces it was often difficult to differentiate respiratory bronchioles from alveolar ducts or the latter from alveoli.

The whole interstitial tissue, and especially the inter-alveolar septa, were extremely infiltrated with numerous large mononuclear cells, lymphocytes, and many plasma cells (Fig. 3).

In sections stained by the Unna-Pappenheim method it was seen that many cells which had not the characteristic morphology of plasma cells had, like the latter, a strongly pyroninophil cytoplasm. No fibroblasts and no collagenous fibres were seen in the interalveolar septa. Although the interstitial cellular infiltration was diffuse it varied considerably in extent in various areas, which explains the patchy appearance on macroscopic examination.

The honeycombed material which filled the terminal air spaces was pale pinkish-violet in sections stained with haematoxylin and eosin, and in sections stained with Giemsa it was clearly seen to consist of closely packed rounded bodies, about 5 μ in diameter, which showed a linear violet margin, a colourless area, a pale purplish central part, and one or two blackish-violet granules or short rods situated eccentrically or close to the margin (Figs. 3 and 4). This structure was equally well recognizable with Heidenhain's azan stain, when the outlines were purple, the granules dark purplish-red or bright red while the material which surrounded the granules stained pale sky-blue. Even better was the structure demonstrable by the McManus–Hotchkiss method, which has been recommended by Hamperl (1952) for this study. With this technique the outline and the granules were bright red, and often within a colourless area a bright red or pink spot was seen. With Masson's trichrome the material stained greenish-blue and with van Gieson's stain greyish-brown or yellowish-brown. Distinct pyroninophilia of granules, spots, and margins was seen in sections using Unna-Pappenheim's stain. No fibrin was found in the material. By the coupled tetrazonium reaction of Danielli (1947), which demonstrates proteins by virtue of their contained tyrosine, tryptophane, and histidine, the honeycombed masses stained pale yellow, while the granules and short rods contrasted by their bright cherry-red colour. The same, though weaker, reaction was seen after benzoylation. It will be shown later that granules and rods represent protozoal chromatin and it is known that nuclear chromatin is comparatively refractory to benzoylation. This was originally attributed to the presence of purine- and pyrimidine-containing compounds. However, according to Pearse (1953) the coupled tetrazonium reaction is always due to the presence of the three previously mentioned aromatic amino-acids, and their relative resistance to benzoylation is the result of " physical " causes inherent in the spatial arrangement of the polypeptide chains of the proteins containing them making them susceptible to some kind of rearrangement through mild heat.

The interpretation of the positive P.A.S. reaction was somewhat difficult, probably because only material fixed with Orth's fluid was available. Not only was the plasmal reaction with $HgCl_2$ oxidation positive, but also in the control preparation, in which untreated sections were placed in leucofuchsin, the material was stained bright red. This reaction was completely blocked by hydroxylamine, thus showing the presence of aldehydes, either in the original material or formed from acetalphosphatides by the action of the fixative which contains bichromate. It was therefore a surprise that prolonged (24 hours) acetylation with acetic anhydride in pyridine blocked completely the P.A.S. reaction. I would suggest that the explanation is to be found in a Canizaro reaction taking place during the prolonged acetylation of the material. Two molecules of pre-formed aldehyde yield one molecule of alcohol and one molecule of carboxylic acid. The former is then blocked by acetylation, the latter is intrinsically P.A.S. negative. It remained to decide whether 1-2 glycol groups were present in the honeycombed material in addition to pre-formed aldehydes. For this purpose the latter were blocked by hydroxylamine, a control section placed in leucofuchsin and another submitted to the P.A.S. reaction. The latter was strongly positive, and, all lipid stains being negative, it may be concluded that the material in addition to aldehydes also contained compounds with 1-2 glycol groups. As mentioned before, the granules and rods which gave a strong P.A.S. reaction represent protozoal chromatin. For comparison the P.A.S. reaction and acetylation were therefore performed on toxoplasma and the result was identical.

The material did not stain with mucicarmine. With Reinhart's modification of Hale's dialysed-iron method a patchy accumulation of acid mucopolysaccharides was demonstrable in some air spaces, but the actual honeycombed material did not stain. With the methylene blue-extinction method, according to Pearse (1953), granules and outlines were stained from *p*H 3.88 upwards ; the whole material was slightly stained at *p*H 5.32 and

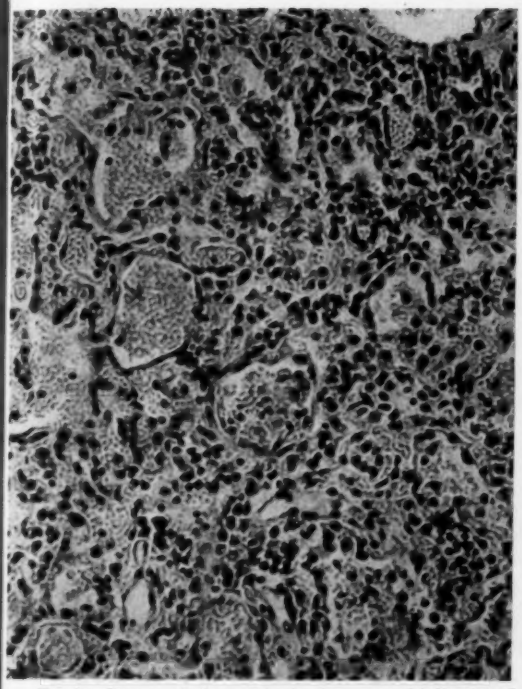

FIG. 1. — Honeycombed masses in the air spaces and infiltration of alveolar septa. Haematoxylin and eosin, × 150.

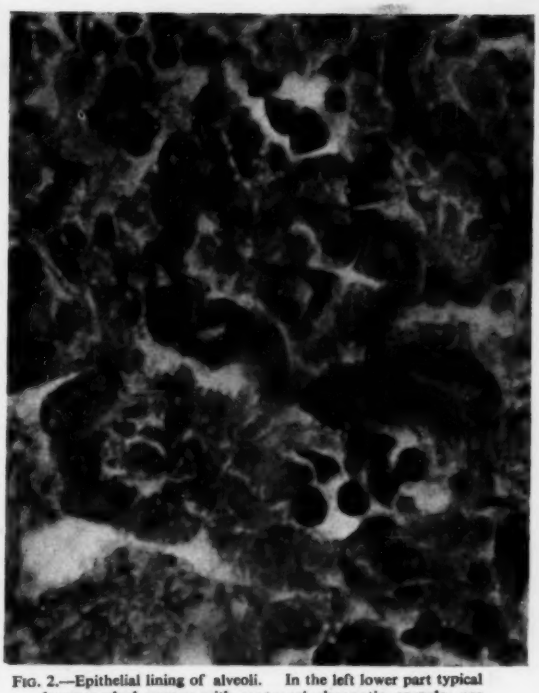

FIG. 2.—Epithelial lining of alveoli. In the left lower part typical honeycombed masses with protozoal chromatin granules are seen. Giemsa, × 625.

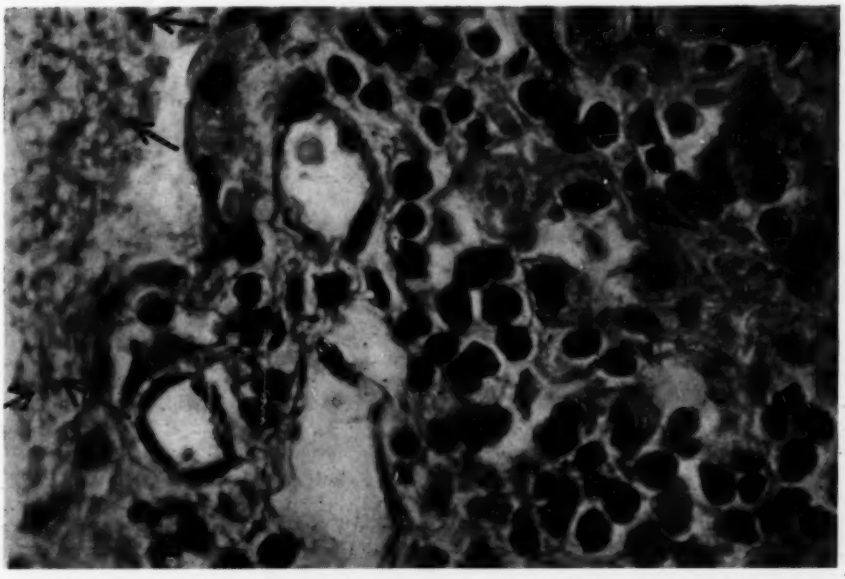

FIG. 3.—The cellular infiltration of interalveolar septa. The arrows on the left side point to protozoal chromatin in a honeycombed mass within an alveolus. Giemsa, × 625.

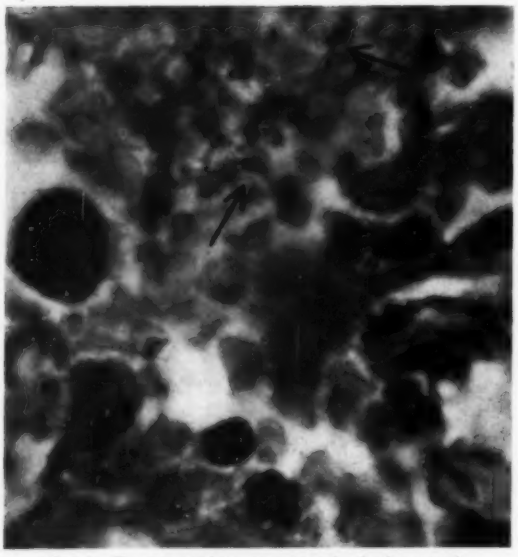

FIG. 4.—The arrows point to granules and rods of protozoal chromatin. Giemsa, × 1,350.

distinctly from *p*H 6.99 upwards. The positive stain at *p*H 3.88 was, in view of the pyroninophilia, almost certainly due to ribonucleic acid. Treatment with ribonuclease (1 mg./ml. for two hours at 37° C.) completely abolished pyroninophilia and almost completely methylene blue, staining at a low *p*H. The same result was obtained by hydrolysis with N/1 HCl at 60° C. for 10 minutes, according to Lajtha (1954). Only embedded material was available for investigation, and so only alcohol-fast metachromasia could be tested and this was negative.

Summarizing the results of staining and histochemical reactions it can be concluded that the honeycombed material consisted of globular bodies of hydropically swollen mucoproteins with circumscribed accumulations of ribonucleic acid. Within the mucoproteins were granules and rods of chromatin which contained large amounts of carbohydrate and ribonucleoproteins and in Orth-fixed material aldehydes which were either pre-formed or resulted from bichromate oxidation of acetalphosphatides.

This description of histological and histochemical findings shows clearly that plasmacellular interstitial pneumonia is entirely different from other forms of interstitial pneumonia of infants, such as the syphilitic pneumonia alba, Hecht's giant-cell pneumonia, and the more common monocytic interstitial pneumonia.

Apart from the pulmonary changes the only histological finding of interest was the presence of typical cytomegalic inclusions in the duct-cells of the parotid and submaxillary glands (Fig. 5). No inclusions were found in the bronchial epithelium or in any other organ, none of which showed any significant changes.

Discussion

Although many authors have in the past remarked on the peculiar honeycombed material in the air spaces in interstitial plasmacellular pneumonia, it was usually considered to be coagulated oedema fluid with cellular debris, and no further attention was paid to this feature until Vaněk and Jírovec in 1952 elucidated the nature of this material by the study of fresh lung-imprints. Characteristic bodies were found which on morphological grounds were identified as the protozoon *Pneumocystis carinii*. This organism was disovered by Chagas in the lungs of guinea-pigs which were infected with *Trypanosoma cruzi*. Chagas believed it to be a stage in the life cycle of trypanosoma, but later authors, such as Carini and Maciel (1915) and Delanoë (1912), showed that it is unrelated to the trypanosoma cycle. Wenyon (1926) places it in the class of sporozoa, subclass coccidiomorpha. He states: "They undoubtedly represent the schizogony stage of some sporozoon, and Stevenson suggests they may be schizonts of Klosiella." This place in the system of protozoa would make pneumocystis related to toxoplasma. Vaněk and Jírovec (1952) classify the organism as a primitive haplosporidium, a well-known representative of which species is the rhinosporidium.

All attempts up to the present time to culture the organism have been unsuccessful and so have animal inoculations. Vaněk and Jírovec's findings were confirmed by Hamperl (1952), by Herzberg, Herzberg-Kremmer, and May (1952), by Bachmann (1953), and partly by Stopka (1952). Comparison of our histological sections with photomicrographs of lung-imprints reproduced in the publications of Vaněk and Jírovec and of Herzberg *et al.* leaves scarcely any doubt that the honeycombed material in the air-spaces represents masses of the protozoa which were identified by the Czech authors. If in addition it is borne in mind how characteristic and different from any type of interstitial pneumonia is the histopathology of interstitial plasmacellular pneumonia, the assumption that *Pneumocystis carinii* is the causative organism appears reasonable although only one of the three postulates of Robert Koch has been fulfilled. Hamperl

(1952) found the typical honeycombed structures in all seven cases which he examined. Herzberg *et al.* (1952) reported that the only case in which they failed to find pneumocystis in lung-imprints presented a different histopathology. Bachmann (1953) found pneumocystis in all of 14 cases of plasmacellular pneumonia which he examined. interstitial plasmacellular pneumonia have been reported up to the present time ; it is also common in this country, where the present case is the first one of interstitial plasmacellular pneumonia to be reported. Cytomegalic inclusion disease is as a rule congenital, transplacentally transmitted ; recently we found a generalized cytomegalic inclu-

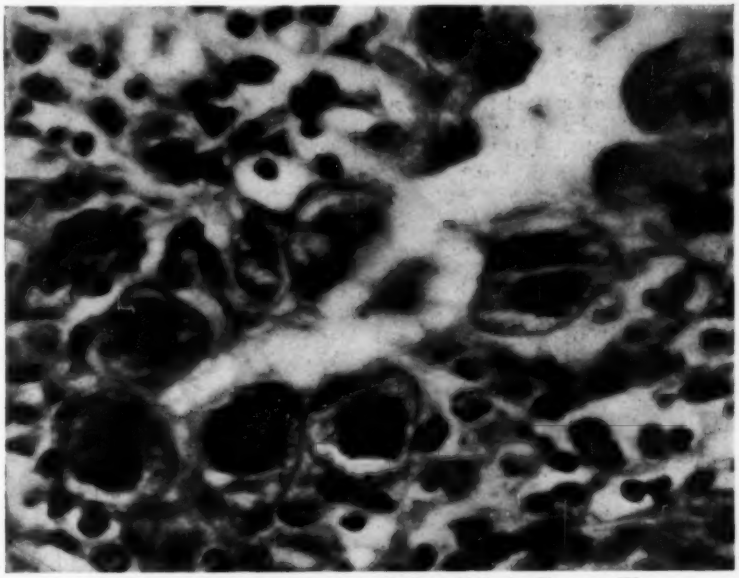

FIG. 5.—Cytomegalic inclusions in duct cells of parotid gland. Giemsa, × 650.

Stopka's report (1952) that out of 17 cases typical foam structures were found only in eight and occasional suspicious appearances in two is probably due to confusion of plasmacellular with other forms of interstitial pneumonia. No histological findings were reported by this author. The same author, in a recent examination of five acute cases by the imprint method, found pneumocystis in all.

An interesting finding in our case is the association with cytomegalic inclusions in salivary glands. Since such inclusions were found in about 5% of the post-mortem material of Birmingham Children's Hospital it was at first considered that the association was coincidental. Recently, however, Dittrich and Seifert (1953) reported this association in 20 examples of interstitial plasmacellular pneumonia. Such a frequency rules out a chance association. For the understanding of the relationship of these two findings the following facts appear to be significant. Cytomegalic inclusion disease is common in the U.S.A., where no examples of sion disease in a macerated foetus. On the other hand, all features of interstitial plasmacellular pneumonia indicate a post-natal infection. No interstitial plasmacellular pneumonia was observed in any of the fatal cases of generalized cytomegalic inclusion disease, nor were cytomegalic inclusions seen in the bronchial epithelium of our case or of any other example of interstitial plasmacellular pneumonia. It is therefore clear that the two conditions are due to different causes. Cytomegalic inclusion disease of salivary glands is present before infection with *Pneumocystis carinii* takes place, and the reasonable explanation appears to be that the symptomless cytomegalic disease which is limited to the salivary glands predisposes to manifest interstitial plasmacellular pneumonia after infection with *Pneumocystis carinii* in a similar way as prematurity and marasmus do. Without such a predisposing factor the pneumocystis infection may remain symptomless. Thus the protozoon was frequently found in a variety of apparently

healthy laboratory animals, in a man who died of Chagas' disease, in a 3-month-old infant with congenital heart disease, in a 4-month-old baby, and a 21-year-old man who died of unrelated disease (van der Meer and Brug, 1942).

In view of considerable evidence in favour of *Pneumocystis carinii* being the causative organism of interstitial plasmacellular pneumonia, the name parasitic pneumonia suggested by Vaněk and Jírovec appears justified.

The geographic distribution of the disease is puzzling, because the occurrence of *Pneumocystis carinii* in animals has been reported from various countries, and in Great Britain it was found by two authors (Coles, 1914 ; Porter, 1916).

The diagnosis is made from clinical symptoms and radiological findings. Pneumocystis has not yet been found in retropharyngeal swabs, but further investigations in this direction are advisable because the infection is resistant to chloromycetin, aureomycin, and other antibiotics, while it appears to respond favourably to simultaneous treatment with " plasmochin " and " atebrin " (Dvořák and Jírovec, 1953).

Summary

A case of interstitial plasmacellular pneumonia is described—the first to be reported outside the European continent. The finding of characteristic honeycombed masses in the air spaces, which were identified by Vaněk and Jírovec as the protozoon *Pneumocystis carinii*, is confirmed. Histochemical reactions of the honeycombed masses are reported. The case was associated with cytomegalic inclusions of salivary glands, and the significance of this association is discussed.

This case was under the care of Dr. Frances Braid. I wish to thank her for the clinical notes. Thanks are also due to Mr. A. R. Detheridge for technical assistance and to Mr. J. G. Williamson for the photomicrographs.

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THE CELLS OF NORMAL HUMAN URINE

A QUANTITATIVE AND QUALITATIVE STUDY USING A NEW METHOD OF PREPARATION

BY

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Despite the many reports of the finding of malignant cells in various body fluids, dating back to Beale, of London, in 1860, the use of exfoliative cytology as a diagnostic procedure did not find much application until the publication in 1943 of the monograph by Papanicolaou and Traut on the diagnosis of uterine cancer by examination of the vaginal smear. Following this classical work the principle has been applied to many regions of the body and now finds widespread acceptance, particularly in the United States, as an aid to diagnosis and for screening in cancer-prevention clinics.

In a survey of the status and future trends of exfoliative cytology Papanicolaou (1952) emphasized the need for more basic studies of the normal cell population in the various body fluids. Of the diverse types and several origins of the cells in urine relatively little is known, although pathological samples have been examined on a large scale. Papanicolaou's paper in 1947 on the examination of samples from 240 clinical cases was followed by several others (Schmidlapp and Marshall, 1948, 1950; Chute and Williams, 1948; Harrison, Botsford, and Tucker, 1951), and in 1952 Crabbe, reporting from this laboratory, gave the results of using the Papanicolaou technique as a screening procedure for 1,000 workers in the dyestuffs industry who may at one time or another have been exposed to bladder carcinogens.

The Papanicolaou technique consists essentially of smearing the sediment from centrifuged urine over an albuminized slide, and, after fixation and staining, the whole smear, which covers most of the slide, is examined under the microscope. The latter process is both laborious and time-consuming owing to the very wide dispersion of the cells. A 50-ml. sample of normal urine produces a quantity of deposit on centrifugation requiring about five to 10 slides to accommodate it when smeared. It is apparent that if the cells could be concentrated on a slide within an area of a few square millimetres the examination could be carried out in a very short time. In addition, the diagnosis would be strengthened by the fact that it would be based upon virtually the whole cell content of the sample rather than a fortuitous small fraction of the whole represented by one or two smears.

In the present paper a method for the concentration of the cells of urine is described and the results of its application to quantitative and qualitative study of the cell population of normal male urine are given.

Technique

The sediment which is deposited on centrifuging normal urine consists largely of mucus.* Removal of this mucus permits free suspension of cells and enables a high degree of concentration to be achieved by centrifugation. A simple procedure is described below by which this may be effected: it depends upon the fact that adding absolute alcohol to the sediment results in fixation of cells without irreversibly precipitating the mucus. It then becomes possible to dissolve the mucus by diluting with water without fear of disrupting the cells.

Determination of the Rate of Cell Loss into the Urine.—(1) About one and a half hours' output of urine in a 70-ml. centrifuge tube marked as shown (Fig. 1) is adjusted to *p*H 6 by adding N/2 HCl or N/2 NaOH and centrifuged for 10 minutes at 1,750 g.† (2) The liquid is gently drawn off and 15 ml. absolute alcohol added and mixed thoroughly. After five minutes distilled water is added slowly with shaking to fill the tube. (3) After centrifugation for 10 minutes at 1,750 g. the liquid is drawn off and about 1 ml. benzidine/hydrogen peroxide solution is added to

* The term "mucus" is used throughout this paper to mean the whole of the mucilaginous material of the normal urinary sediment, and is broadly justified by the protein reactions of the cell-free sediment and by the action of 33% acetic acid on an aqueous solution of this sediment (see Harrison, 1947).

† Centrifugal force is expressed throughout as a multiple of normal gravity (g.).

"stain" erythrocytes, and well mixed. The centrifuge tube is stoppered and left for half an hour. (4) Dilution with about 30 ml. 20% alcohol is followed by centrifugation for 10 minutes at 1,750 g. and the liquid drawn off to leave about 1 ml. (5) Cell clumps are broken up by drawing the suspension vigorously in and out of a pipette connected to a hypodermic syringe. (6) The suspension is now centrifuged again for five minutes at 1,750 g., the liquid is drawn off to either 0.05 or 0.1 ml., and cell counts are carried out using a Neubauer chamber.

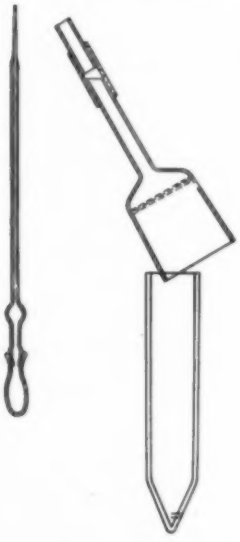

FIG. 1.—The main items of apparatus used, shown at about a quarter actual size. The 70 ml. centrifuge tube comes internally to a fairly sharp point, and is marked at 0.05 and 0.1 ml. The modified Pasteur pipette, used for handling the concentrated cell suspension, should have an internal diameter of 0.5 mm. in the terminal drawn-out portion. The Buchner funnel, containing a sintered glass filter of pore size 2, is used when it is desired to separate large cells from small cells. The mouthpiece is added before blowing through the filter.

Areas of 1 to 4 sq. mm. are then counted for large cells, small cells, and erythrocytes. An arbitrary value of 15 μ diameter is taken to define large and small cells: in practice the sizes of most of the cells are found to fall conveniently far from this. The benzidine treatment shows erythrocytes as black discs. The calculation is thus: Cells counted $\times \frac{V}{vT}$ = loss in thousands/hour, where V = volume of suspension in ml. (0.05 or 0.1), v = volume of suspension actually counted in c.mm., and T = period of secretion of urine in hours. The time taken for two determinations in parallel is about three hours.

Preparation and Staining of Cellular Contents of Urine.—Stages 1 and 2 given above are carried out. Then (3) crystals and the grosser "dirt" particles (and unavoidably a few cells) are deposited by centrifuging for one minute at about 280 g., and are then removed with a pipette. (4) After centrifugation for 10 minutes at 1,750 g. most of the liquor is drawn off, and one half of the remaining volume of a pectin adhesive solution is added and mixed. After a further centrifugation for two minutes at 1,750 g. liquid is removed until a volume roughly equal to that of the sediment remains. (5) After mixing, the whole is transferred by pipette to a grease-free microscope slide. The diameter of the droplet thus deposited is usually about 2 to 10 mm. As evaporation proceeds, a mat, slightly glistening spot remains on the slide which is placed horizontally in 1:1 alcohol/ether fixative.

It is sometimes convenient to make two preparations, one of the small cells (less than 15 μ in diameter) and the other of the remaining cells. The separation is carried out by means of a sintered glass filter.

If this separation is desired, the following should be carried out after stage 3:

The cleaned cell suspension is poured into a sintered glass filter funnel, pore size 2, the filtrate from which contains only the small cells; this is collected in a 70 ml. centrifuge tube. Meanwhile large cells have been accumulating on the filter; when only a small depth of liquid remains, and before it falls to zero, the filter funnel is inverted over another centrifuge tube, as shown in Fig. 1. A little 20% alcohol is then blown through the filter transferring the cells on it to the centrifuge tube.

Pectin is effective as an adhesive in minute concentration, and being a carbohydrate is relatively refractory to stains.

Staining.—From alcohol/ether the preparation is taken through the following sequence (10 seconds or longer at each stage unless otherwise stated):—

Seventy per cent. alcohol; benzidine-H_2O_2 solution, 10 minutes; 70% alcohol; 50% alcohol; Harris' haematoxylin, 10 minutes; 50% alcohol; 70% alcohol containing 1% NH_4OH, one minute; 70% alcohol; orange G, one minute; absolute alcohol, irrigate to remove all surplus orange G; light green, one minute; absolute alcohol, one second; xylol. The preparations are mounted in balsam.

Small-cell Preparations.—Haematoxylin 5 minutes; counterstains at half strength, or use 0.5% eosin.

For Screening for Malignancy.—Counterstains at half strength.

The time taken to make the general preparation of six urine samples in parallel is two and a half hours. To make separate small-cell and large-cell preparations of each of six samples, about four hours are required.

Composition of Solutions Employed.—The following solutions are employed:—

Pectin Adhesive.
Pectin 0.02%
Glycerin ... 4.0% } in water

Benzidine-Hydrogen Peroxide.
Benzidine ... 1.0%
Acetic acid ... 2.0% } in 96% alcohol

To 50 ml. of this stock solution 1 ml. 20-volume hydrogen peroxide is added. After 24 hours the mixture is centrifuged.

Haematoxylin.
Harris haematoxylin
Alcohol 70% } equal volumes

Orange G.
Orange G ... 5% in 50% alcohol
Light Green.
Light green ... 0.2% in 96% alcohol

The Rate of Cell Loss into the Urine

Using the method described above, the rate of cell loss has been determined on 12 men of ages varying from 18 to 47, none of whom was aware of any previous history of kidney, bladder, or prostatic complaints or of macroscopic haematuria. Part or all of a two-hour sample was used, secreted during the period 7.30 a.m. to 9.30 a.m. in each case. Subjects were asked to ensure that the urine stream did not come into contact with the prepuce. Table I shows the rates of cell loss obtained.

TABLE I
RATE OF CELL LOSS IN THOUSANDS/HOUR

Subject No.	Squamous, Transitional, and Degenerate Cells	Small Epithelial Cells and Leucocytes	R.B.C.s (1,000/hour)
I	6·2	34·0	2·8
II	31·1	76·6	13·0
III	10·1	54·6	3·7
IV	33·0	177·1	1·5
V	14·8	46·7	0·1
VI	74·8	88·3	0·8
VII	148·9	165·0	3·7
VIII	31·6	62·3	1·1
IX	45·2	28·2	1·2
X	21·6	136·6	1·0
XI	17·3	56·6	0·1
XII	12·2	206·6	2·8

It appears from this limited series that the output of blood cells and exfoliation proceed at very different rates in different individuals. In the same individual, however, the output appears to be constant from day to day. The variation in total cell output as indicated by a repeat determination on another day on each of the 12 subjects was from 0.5 to 20.5%.

The results of a preliminary assessment of diurnal changes in the rate of cell loss in one individual is shown in Figs. 2a and 2b. The red cell output appears to be increasing with the day's activities. The peaks occurring in Fig. 2b for the period 6–8 a.m. might well be due to sloughing off of cells which have accumulated in the upper layers of the epithelium during the night.

The Normal Urine Cell Picture

Samples were obtained from the same subjects. All urines were produced between 7.30 a.m. and 9.30 a.m. By means of the technique described, the cells were separated from extraneous matter and segregated into two groups, those greater than about 15 μ diameter (mainly squamous and transitional) and those less than about 15 μ diameter.

FIGS. 2a and 2b.—Diurnal changes in the rate of cell loss from the urogenital system. In this preliminary experiment the changes occurring in one individual on two days (broken line and continuous line) are indicated. Fig. 2a refers to red blood cells. Fig. 2b refers to large epithelial cells and to small epithelial cells and leucocytes.

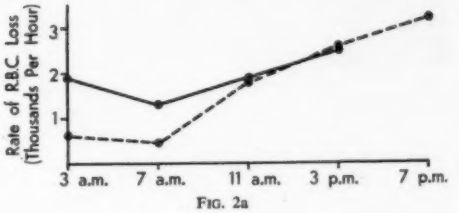

FIG. 2a

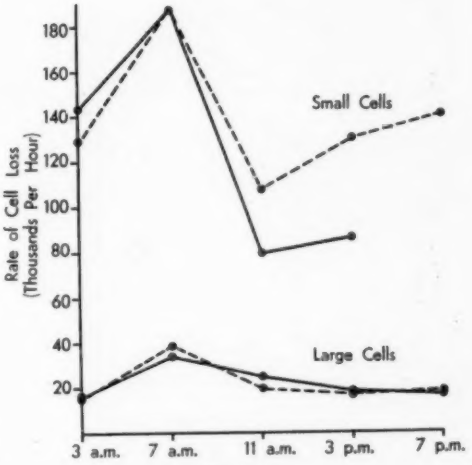

FIG. 2b

They were then mounted and stained. Figs. 3 and 4 show typical fields of these large-cell and small-cell preparations.

The Large Cells.—Examples of the main types of cells to be found in the large-cell preparation are shown in Fig. 5. Table II shows that the proportions of the various kinds of large cells exfoliated vary greatly between individuals, although, so far as this has been studied, there appears to be remarkable consistency in the cell picture of urine from the same subject at different times.

The figures in Table II were obtained indirectly by carrying out differential counts on stained preparations of the large cells of normal male urine and applying these proportions to the directly obtained rate of large-cell loss for each individual given in Table I, column 2. About 250 cells were

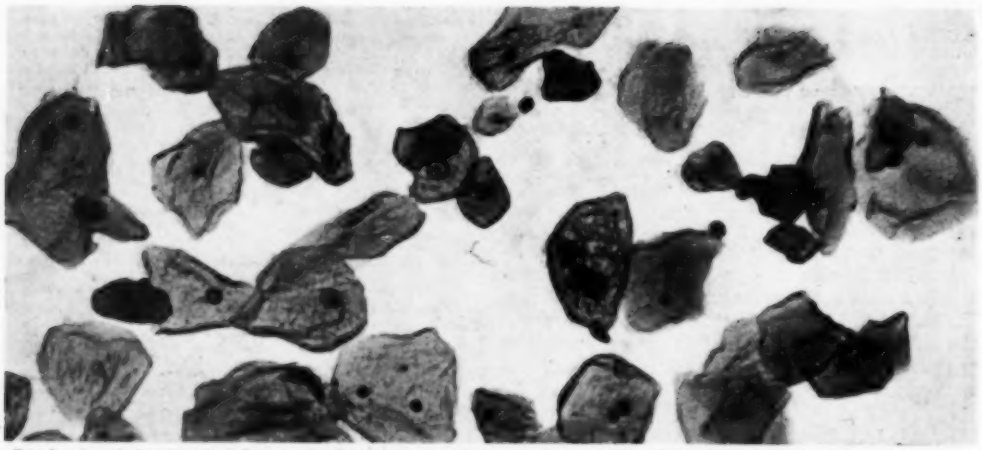

FIG. 3.—A typical preparation of large epithelial cells from normal male urine. × 350. Cells smaller than about 15 μ diameter have been removed by filtration.

counted in each case. The last two columns show the reaction to staining with 5% orange G followed by 0.2% light green.

The individuality of the cell picture is evident in one or more of the following: (i) the total quantity of cells present (see Table I); (ii) the occurrence of multinucleate cells (see Fig. 5); (iii) the relative proportions of squamous, transitional, "early degenerate," and "late degenerate" cells (see Fig. 5); and (iv) the reaction to staining with 5% orange G followed by 0.2% light green (see Table II).

All of these differences invite speculation, not least the reaction to orange G and light green. For example, Subject II shows 91.9% of the cells stained green, Subject XI shows 76.0% stained yellow. The cells which stain yellow are mainly the degenerated type: a count of 973 yellow-staining cells from 20 normal male subjects shows that 2.1% are squamous, 3.7% are transitional, 16.8% are "early degenerate" cells, and the remainder, namely 77.4%, are "late degenerate" cells. These figures are in accord with the preferential staining of cornified tissues by orange G. The

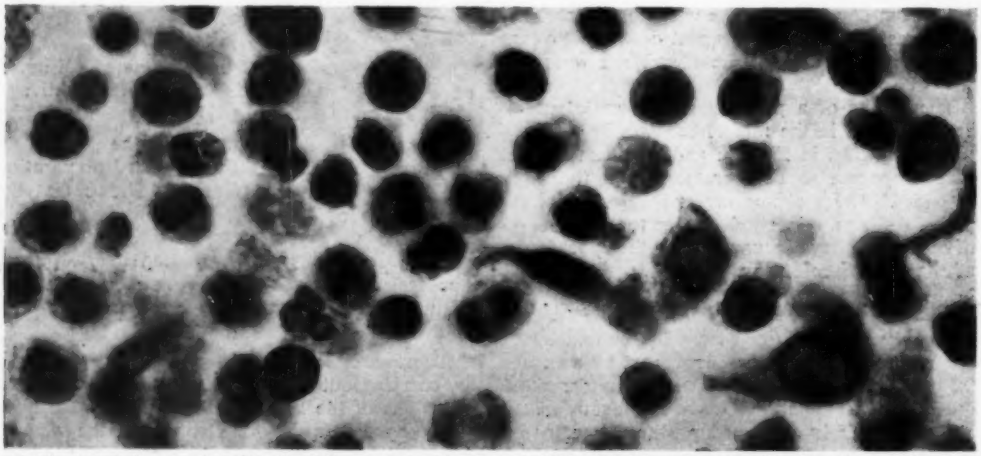

FIG. 4.—A typical preparation of small cells from normal male urine. × 1,750. Cells larger than about 15 μ have been excluded by filtration. A clearly defined red blood cell is seen bottom left.

TABLE II
RATE OF LARGE CELL LOSS IN THOUSANDS/HOUR

Subject No.	Transitional	Squamous	Early Degenerate	Late Degenerate	Staining Reaction	
					% Green	% Yellow
I	1·9	1·1	1·7	1·5	67·7	32·2
II	11·2	12·1	6·3	1·5	91·9	8·1
III	2·2	1·1	1·7	5·1	41·2	58·8
IV	12·4	3·6	15·8	1·1	70·5	29·5
V	3·9	5·5	3·6	1·9	77·1	22·9
VI	10·5	5·0	30·0	29·2	61·0	39·0
VII	20·6	31·5	77·0	19·8	83·3	16·7
VIII	16·4	7·6	1·0	7·3	74·6	25·4
IX	26·2	3·5	4·0	11·3	79·6	20·4
X	9·3	1·6	2·2	8·6	52·9	47·1
XI	2·8	1·0	1·7	11·8	24·0	76·0
XII	4·1	1·3	0·7	6·0	43·5	56·5

common occurrence in some subjects of apparently undegenerate squamous and transitional cells which nevertheless give a strong yellow reaction could perhaps be taken to show that this reaction is a more sensitive indication of degenerative changes in these types of epithelium than structural criteria. A further point of interest is the occurrence of groups consisting of about six or more structurally identical cells which have developed together and peeled off en masse, in which one or two cells show a strong yellow reaction,

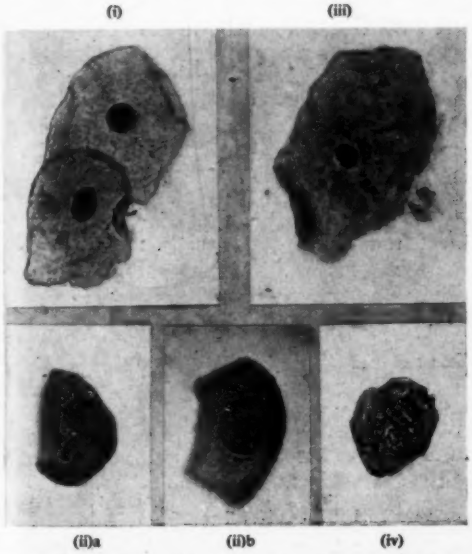

FIG. 5.—The cell types common to all preparations of the large cells of normal male urine. × 495. (i) Squamous epithelial cells. (ii)a and (ii)b Transitional epithelial cells. (iii) "Early degenerate" cell: a squame in the early stages of degeneration: the nucleus is pyknotic. (iv) "Late degenerate" cell. The nucleus has disappeared and the cytoplasm has a crinkled appearance. Figures for the rates at which these cells are lost are given in Table II.

although all their fellows stain strongly green. It is tentatively suggested that there is a relation between the state of degeneration of the cells and their affinity for the yellow stain, but signs of degeneration may be difficult to recognize morphologically in some cases.

The Small Cells.—Examples of the main types of small cells are shown in Fig. 6. Table III gives some indication of the rates at which they are lost into the urine: it is evident that there is great variability between individuals.

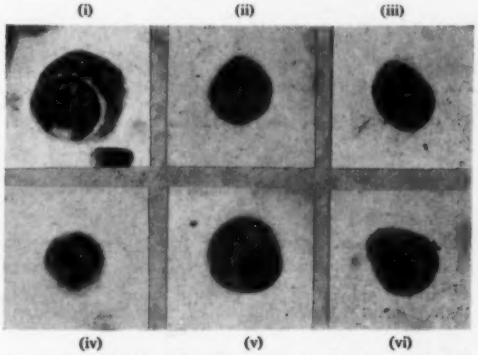

FIG. 6.—The principal cell types common to all preparations of the small cells of normal male urine. ×1,550. Evidence is submitted suggesting that type (i) originates in the kidney; (ii) is probably a degenerate form of one or more of the other types; (iii), (iv), and (v) represent comparatively well-defined examples of a group which includes numerous types intermediate to these; (vi) is a polymorphonuclear leucocyte. Figures for the rates at which these cells are lost are given in Table III.

The figures in Table III were obtained indirectly by carrying out differential counts on stained preparations of the small cells of normal male urine and applying these proportions to the directly obtained rate of small-cell loss for each individual given in Table I, column 3. The average number of cells counted in each case was 280. Rates for loss of red cells are repeated from Table I.

TABLE III
RATE OF SMALL CELL LOSS IN THOUSANDS/HOUR

Subject No.	Cell Types (Fig. 6)			Polymorphs	R.B.C.s
		ii	iii, iv, v		
I	3·6	17·1	13·3	—	2·8
II	5·1	55·8	15·8	—	13·0
III	5·5	17·2	21·0	10·8	3·7
IV	2·4	20·9	22·5	131·5	1·5
V	1·9	29·0	9·8	6·0	0·1
VI	6·1	32·0	32·0	18·9	0·8
VII	1·4	29·3	19·7	123·9	3·7
VIII	1·8	10·4	7·1	43·1	1·1
IX	0·6	15·5	8·9	3·1	1·2
X	5·4	43·9	57·5	29·8	1·0
XI	1·5	10·2	7·0	37·0	0·1
XII	8·8	53·0	38·8	106·0	2·8

FIG. 7a.—Application to cytological diagnosis. Urine from a case of carcinoma of the bladder. × 660. A smear of the urinary sediment was prepared according to the standard method of Papanicolaou. Many fields showed no cells; this field showed the most cells out of 15 fields taken at random. For comparison with Fig. 7b.

The following criteria were used in counting. Cell type i: a very round granular nucleus and high nuclear/cytoplasmic ratio; cell type ii: no nucleus visible, granular cytoplasm, sometimes vacuolated. The cell types designated iii, iv, and v in Fig. 6 have not been found to be sufficiently characteristic to permit individual classification: they have therefore been counted together, along with the various intermediate types which occur.

Counts of cells of 8 to 30 μ diameter to be found in fresh urinary sediment have been carried out for many years by Addis (1948), who gives 650,000/24 hours as the normal rate of loss of epithelial and white blood cells, and assumes that most of the epithelial cells are derived from the kidney. In the present study a preliminary attempt has been made to indicate the origin of the cell types referred to above by making routine preparations (Section II) of the following material: (1) macerated normal human kidney tissue suspended in cell-free urine and set aside for an hour; (2) urine removed from a human ureter at operation; (3) normal female urines. In addition (4) smears were made of prostatic fluid of three subjects. In (1) and (2) most of the cells were similar to the cells referred to above as type i. A few of these were found in the female urines and none in the prostatic smears. It thus seems reasonable to consider that the type i cells of urine have originated in the tubules of the kidney.

The type ii cells are very probably degenerate forms and are often found to be breaking up. The numbers in which they occur are not connected with deterioration of the urine sample, as when fresh samples are used the output is consistent from day to day at the level characteristic

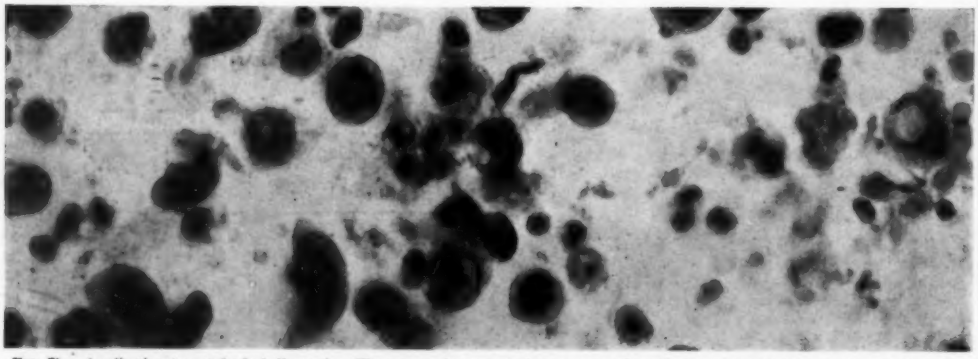

FIG. 7b.—Application to cytological diagnosis. The same urine sample from a case of carcinoma of the bladder as shown in Fig. 7a. × 660. The urine was treated according to the short method advocated. A field taken at random.

for the individual. The fact that they hardly ever stain preferentially with orange G indicates that the cells of which they are degenerate forms do not originate from a region in which cornification occurs.

The invariable presence of red cells in all individuals is of interest (see Table III), particularly as the figures given represent the minimum number present. Ghosts and broken red cells were not included. All those counted were unmistakably identified by the benzidine reaction. The mode of entry of blood cells into urine presents an interesting problem. The greater ability of polymorphs to pass from the blood into urine is demonstrated by the change of the R.B.C./polymorph ratio from about 1,000/1 in blood to about 1/30 in urine.

Application to Cytological Diagnosis

Mention has been made of the ease with which the whole cellular contents of a sample of urine may be examined, and of the possibility of improved accuracy in diagnosis thus presented. Figs. 7a and 7b give some idea of the appearance of preparations made from the same sample by the smear technique and by the method described above.

It seems possible that the principle of fixation of cells followed by solution of extraneous material may be applied with advantage to other body fluids or washings used for cytological diagnosis. The use of mechanical segregation by means of a filter may also find application where it is desired to examine cells of a particular size occurring in small proportions, and would be of value where pus, blood, or bacteria are crowding out the cells under examination.

With regard to renal diagnosis, Foot and Papanicolaou (1949) have shown that single cells of the tubule epithelium become malignant, though their fellows appear normal and the lumen remains patent. If, as is now submitted, the cells of the small-cell preparation contain identifiable renal cells, there seems a reasonable chance that its use might enable early identification of malignancy in the kidney.

Summary

A method is described whereby the number and type of cells in a sample of urine may be accurately determined. The method depends upon the solution and removal of mucus after the urine sediment has been treated with absolute alcohol. The cells may then be concentrated by centrifugation and counted by means of a standard blood counting chamber, or they may be segregated mechanically according to size and mounted and stained for microscopic examination.

The use of pectin as an adhesive is described, and staining procedures are given.

The method was used to investigate the rates of exfoliation of epithelial cells and of the loss of leucocytes and erythrocytes into the urine in 12 normal males. The results of a preliminary experiment on the diurnal changes in the rate of cell loss are also given.

An indication of the normal urinary cell picture has been established by differential counts on stained preparations of the cells from the same 12 subjects.

Evidence is submitted suggesting that the origin of certain of the small cells of the urinary sediment is the epithelium of the kidney.

The possible application of the method to cytological diagnosis is briefly discussed.

I wish to record my grateful thanks to the head of these laboratories, Dr. M. W. Goldblatt, at whose instance the work was undertaken, and who assisted in the preparation of this paper; also to Dr. J. G. S. Crabbe, whose wide experience of exfoliative cytology has been most helpful.

For the invaluable assistance of Miss A. C. Metcalf and for the care and skill of Mr. K. D. Cooper in the preparation of the photographs, I am most grateful.

I am greatly indebted to the Royal Cancer Hospital for making available operative material and to Mr. D. M. Wallace, consulting urologist, for his interest and most generous cooperation in various aspects of this work.

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A CASE OF CRYPTOCOCCAL MENINGITIS

BY

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Cryptococcosis is a disease caused by the fungus *Cryptococcus neoformans*, one of the yeast-like organisms. The disease is world-wide in distribution, but most cases have been reported from the U.S.A. and Australia (Cox and Tolhurst, 1946). Some 200 cases are reported in the American literature (Carton, 1952 ; Evans and Harrell, 1952).

The interest of the case reported lies in its apparent rarity in this country, only 10 cases of cryptococcosis having been reported to date (Smith and Crawford, 1930 ; Greenfield, Martin, and Moore, 1938 ; Blair, 1943 ; Magarey and Denton, 1948 ; Daniel, Schiller, and Vollum, 1949 ; Galton and Whittick, 1950 ; Taylor, 1953 ; Symmers, 1953) ; in the demonstration of a possible differential stain for the cryptococcus *in vivo* ; and in the use of a new fungicide in the treatment of the disease.

Cases occur more frequently in men than in women (Evans and Harrell, 1952), as do the other deep mycoses (Ainsworth, 1952). There does not appear to be an especial occupational hazard with this fungus, as with some others (Raphael and Schwarz, 1953 ; Conant, Smith, Baker, Callaway, and Martin, 1954).

The organism has a particular affinity for the central nervous system and causes either a localized granuloma or more often generalized meningitis (Daniel *et al.*, 1949). Occasionally the fungus remains localized in the lung, which is believed by Conant *et al.* (1954) to be the portal of entry.

As regards the possible source of infection, Emmons (1951) isolated from the soil four strains virulent to mice and with typical fermentation reactions.

Carter and Young (1950) found pathogenic cryptococci in milk. Non-pathogenic cryptococci are found in the intestinal tract of man and on human skin (Benham and Hopkins, 1933). In this laboratory Dr. W. D. Nicoll (1954), examining stored blood from the hospital blood bank, found two bottles from over 300 examined contaminated with non-pathogenic cryptococci.

C. neoformans may be differentiated from the other yeast-like fungi without much difficulty. It differs from *Candida* in its possession, especially on first isolation from the body, of a large gelatinous capsule, and from *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *brasiliensis* as well as *Coccidioides immitis* in its lack of biphasic forms of growth, since it remains in the "yeast-phase" both at 37° C. and at room temperature. *C. neoformans* grows readily on Sabouraud's glucose agar, on blood agar, and in nutrient broth in a few days. On solid media a creamy-white, shiny colony appears initially. Later it becomes honey coloured and shows a tendency to drip down the surface of the slope, again rather like honey. Fermentation reactions are not essential for identification, the other features being quite distinctive. Microscopically, single budding (in this case fungi presented occasional double budding) yeast cells are seen within a large capsule (Figs. 1 and 2). The yeast cell has a double outer wall and within the cell itself there is a small, ill-defined refractile area. The capsule has been shown to be polysaccharide in nature (Evans and Mehl, 1951) and is serologically active. Evans and Kessel (1951) were able to distinguish three antigenic types. The capsule becomes smaller on culture, but is still sufficiently apparent to permit differentiation from species of *Candida*.

The organism may be easily identified in cerebrospinal fluid by the addition of india ink or "nigrosin," when the capsule and its contained yeast cell clearly stand out against the black background (Fig. 3). Its presence may be suspected in the counting chamber either from the presence of budding cells, or from the oval shape of the cells and their relatively large size. They can be differentiated from red blood corpuscles, since the latter are refractile when slightly out of focus. If the specimen is spun down, the capsule may be thrown into sharp relief by other cells or debris collected around it (Fig. 4). Staining in the counting chamber by 0.1% toluidine blue gives the fungus a definite pink tinge leaving the capsule unstained, while white blood cells are stained deep blue and red blood cells remain unstained.

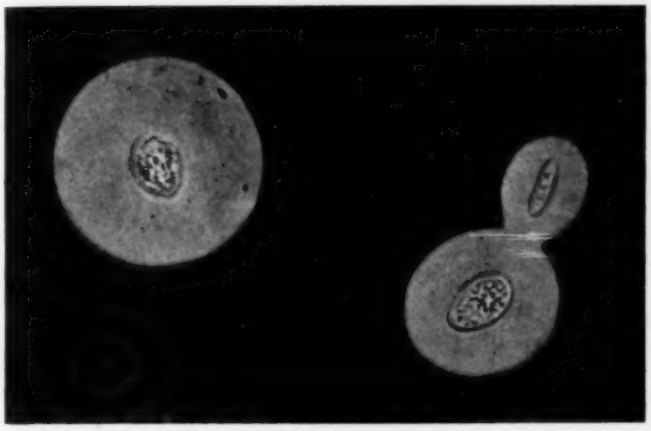

FIG. 1.—Budding and non-budding cryptococci from cerebrospinal fluid. India ink preparation, × 750.

Case Report

A 42-year-old carpenter and joiner was seen at the hospital on May 1, 1953. His chief complaint was pain in the neck and shoulders, of six weeks' duration. His past history was not helpful.

The illness began with an ill-defined infection of the upper respiratory tract followed by constant headache, pain in the back of the neck made worse by walking downhill, buzzing in the ears, and nausea. Later the pain radiated to the shoulders and anorexia appeared.

Physical examination on admission on May 5 disclosed Kernig's sign positive, neck movements restricted, and tenderness over the cervical spine but not over the shoulders. There was diplopia on lateral movement of the eyes in both directions. Some wasting of the left upper arm was noted. The biceps jerk was absent on both sides as was the knee jerk on the left. Plantar responses were flexor. On May 6 lumbar puncture showed cerebrospinal fluid pressure of 250 mm. of water, Queckenstedt's test was normal, 86 cells were found per c.mm. of fluid (15% polymorphs and 85% lymphocytes), protein was 150 mg.%, chloride (as NaCl) 650 mg.%, and globulin in excess. Lange's gold curve was 55432210. The Wassermann test and Price's precipitation reaction were negative.

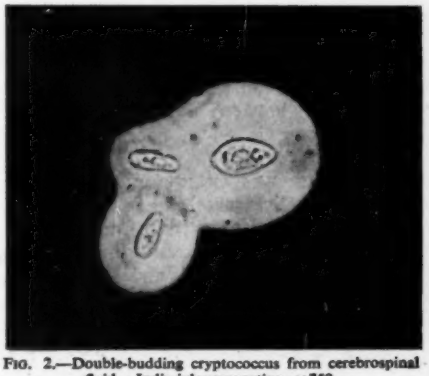

FIG. 2.—Double-budding cryptococcus from cerebrospinal fluid. India ink preparation, ×750.

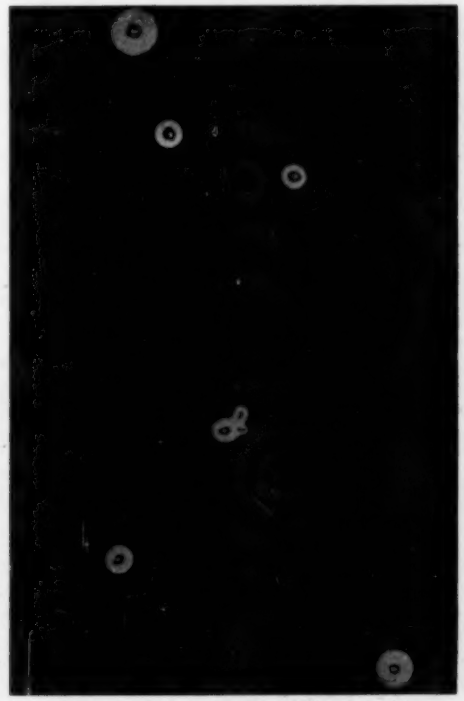

FIG. 3.—Cryptococci in cerebrospinal fluid. India ink preparation, ×180.

On May 13 findings were similar. On May 14 the sugar level was 14 mg.%, and budding cryptococci were found in the cerebrospinal fluid.

On May 15 review of the neurological findings disclosed additional signs. The left pupil had become larger than the right; there was right facial weakness and weakness of flexion and extension of the right arm. Right-sided ptosis was noted on May 24. Meanwhile cultures of the C.S.F. at room temperature and at 37° C. were positive in 48 hours.

Treatment with "actidione" was begun on May 29; 20 mg. was given intravenously for three days, then 40 mg. intravenously daily, and 10 mg. intrathecally on alternate days. On June 9 dosage was increased to 60 mg. intravenously and 20 mg. intrathecally daily. The total amount administered was 720 mg. intravenously and 200 mg. intrathecally. The patient's clinical condition progressively deteriorated, and he died on June 17 with bronchopneumonia. Necropsy was refused.

Cultures of cerebrospinal fluid were positive throughout the course of his illness, although the number of cryptococci per c.mm. varied (Table I).

Radiographs of the chest were reported as normal. The temperature during the period in hospital showed one peak to 100° F. Two blood cultures taken at this time were sterile despite prolonged incubation. Cryptococci were not found in urine or in nasal or throat swabs. The E.S.R. on May 18 was normal.

TABLE I

COUNTS OF CRYPTOCOCCI AND WHITE BLOOD CELLS IN CEREBROSPINAL FLUID

Date	Cryptococci (per c.mm.)	W.B.C. (per c.mm.)
May 20	63	7
" 27	168	8
" 29	240	12
" 31	265	33
June 2	122	22
" 4	195	23
" 6	88	96
" 8	50	55
" 10	31	43
" 12	94	43
" 14	98	44
" 16	47	41

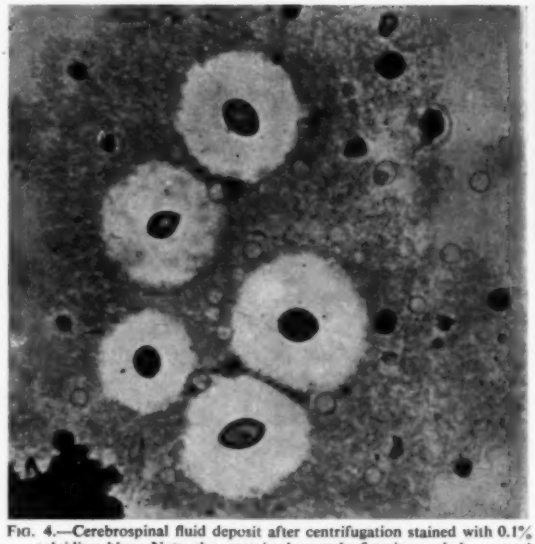

FIG. 4.—Cerebrospinal fluid deposit after centrifugation stained with 0.1% toluidine blue. Note the unstained capsule forming a halo around the ovoid cryptococci against a background of debris and cells, the lack of capsule around lymphocytes, and the unstained red blood cells, ×500.

Bacteriological Investigations

The probable source of infection was the soil. The patient was a carpenter and joiner who worked largely in the open air on building sites. He had been working in one particular area for the previous three years within a radius of one mile from his carpenter's shop. He worked mainly with Canadian fir and pine; occasionally he handled felt and hardboard. Specimens of sweepings from his workshop, from the timber at the building site, of soil around several of the houses on the site, and of hardboard, felt, and sawdust from his bench were obtained. The materials were ground up and emulsified in saline with penicillin (20 units/ml.) and streptomycin (40 μg./ml.) added, and then injected intraperitoneally into white mice. The mice all died within a few weeks, but in none at necropsy was there evidence of cryptococcal granulomatosis. Cultures were taken from peritoneum, brain, and lung in some animals, with similar negative findings. No cause of death was determined.

The fungus and its capsule were measured using a micrometer; the capsule diameter varied between 33 μ and 60 μ (20 cells measured), while the yeast itself measured between 6 μ and 21 μ (average diameter of capsule 44 μ, average diameter of fungus 12 μ).

The organism was tested for its sensitivity to various therapeutic agents. By the disc sensitivity method it appeared insensitive to penicillin, chloromycetin, aureomycin, terramycin, *para*-aminosalicylic acid, streptomycin, and methylene blue. Zones of growth-inhibition were found around discs prepared with acriflavine, crystal violet, isonicotinic acid hydrazide, sulphadiazine, and pentamidine.

On the basis of these tests, tube sensitivity tests were set up with isonicotinic acid hydrazide, pentamidine, crystal violet, acriflavine and sulphadiazine.

A broth culture containing 670 viable organisms/c.mm. was used and the drugs under examination were added to give the required concentrations. Pentamidine was fungistatic in concentrations of 1.56 μg./ml., crystal violet at 150 μg./ml., and acriflavine at 60 μg./ml. Isonicotinic acid hydrazide and sulphadiazine did not inhibit growth.

Shortly afterwards actidione was obtained and tube-sensitivity tests with this agent were set up. Actidione was found to be fungicidal and fungistatic in a concentration of 0.625 μg./ml.; 0.3125 μg./ml. was fungistatic but not fungicidal. Pentamidine under the same conditions was fungicidal at 25 μg./ml., but at 1.56 μg./ml., although fungistatic, it was not fungicidal.

A sample of cerebrospinal fluid which contained 31 cryptococci and 43 white blood cells per c.mm. on June 10 was Seitz filtered. The fluid was then examined again in the counting chamber; no organisms were now present. In each of two test-tubes, 1 ml. of filtered cerebrospinal fluid was placed. To one of these tubes were added three drops of a broth culture of the strain of *C. neoformans* isolated in this case, and to the other three drops of broth culture of a strain of *C. neoformans* provided by Dr. R. W. Riddell. Subcultures of these specimens were positive after incubation, indicating that "actidione" had not reached a fungicidal level in the cerebrospinal fluid. Chemical methods of estimating "actidione" were not available.

Animal Inoculation Experiments

Six animals were injected intraperitoneally with cerebrospinal fluid on May 16, 1953. The results of these injections may best be seen in Table II. Brain, lung, liver, spleen, kidneys, and bowel were examined microscopically in every case, while especial features were examined as they occurred.

It is interesting to note that death only occurred spontaneously in those animals in which the infection spread to the lungs. Where the disease remained localized beneath the skin, the animal survived and appeared healthy despite relatively large cryptococcal tumours beneath the skin.

A second series of inoculations was made from saline-suspended cultures on May 28, and the results are listed in Table III.

TABLE II
FINDINGS IN MICE

Mouse No.	Died	Killed	Period of Survival (Days)	Findings
186/54	—	27/1/54	256	Subcutaneous toruloma only
181/54A	—	27/1/54	256	Subcutaneous toruloma only with calcification of peripheral cryptococci (Fig. 5)
181/54B	—	27/1/54	256	No evidence of disease
181/54C	—	27/1/54	256	Subcutaneous toruloma only
2392/53	17/11/53	—	185	Subcutaneous toruloma and lung cryptococcosis (Fig. 6)
2442/53	19/11/53	—	187	Subcutaneous toruloma and lung cryptococcosis

TABLE III
INOCULATIONS OF SALINE-SUSPENDED SOLUTION IN MICE

Mouse No.	Died	Killed	Period of Survival (Days)	Findings
23/54	5/1/54	—	222	Cryptococci in lungs only (only lungs examined)
24/54	—	5/1/54	222	Toruloma on abdominal wall
183/54	27/1/54	—	244	Focal areas of chronic inflammation in liver and kidneys; no fungi seen

Again it will be noted that localized cryptococcal infection within the anterior abdominal wall was compatible with survival. The lesions in mouse No. 183/54 were interpreted as healed lesions.

The mice in both series survived six months or more, even when cryptococcosis was the cause of death. Other workers have been able to produce fatal lesions in less time. This fact, combined with the production of abdominal wall "torulomata," suggested that inoculations of both cerebrospinal fluid and suspensions of the organism had actually been made subcutaneously and not intraperitoneally. In order to test this theory a further three mice were later given intraperitoneal injections, and a control group intradermal injection, using at

TABLE IV
RESULTS OF INTRAPERITONEAL INJECTION IN THREE MICE

Mouse No.	Died	Killed	Period of Survival (Days)	Findings
182/54	25/1/54	—	10	Cryptococcal perisplenitis and interstitial pancreatitis
185/54	27/1/54	—	12	Cryptococci in brain, kidney, and perisplenic tissues
524/54	9/3/54	—	53	Cryptococci in brain, lung, and abdominal wall

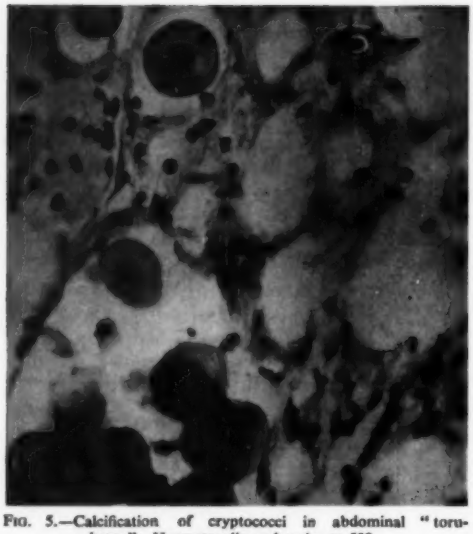

FIG. 5.—Calcification of cryptococci in abdominal "toruloma." Haematoxylin and eosin, × 500.

the same time a heavier inoculum (40 million organisms to each mouse). The results appear in Table IV.

These results strongly suggested that the earlier inoculations had not indeed been made intraperitoneally. This is confirmed by the fact that the three control mice injected intradermally are still alive.

The histological appearances were similar to those previously described (Conant *et al.*, 1954; Symmers, 1953), and the lack of cellular reaction to the cryptococcal bodies was striking (Fig. 6) except in the liver and kidney, where there was a mild inflammatory response. Specific staining methods showed that the fungi gave a positive reaction to the periodic-acid-Schiff method, to Meyer's mucicarmine, and to Gram's stain. Metachromasia was observed with methylene blue and crystal violet.

Discussion

Cryptococcal meningitis is not difficult to diagnose in the laboratory once the possibility has occurred to the examiner of the cerebrospinal fluid. It is quite possible for cryptococci to be mistakenly identified as lymphocytes or red blood corpuscles, but if the true nature of the bodies is suspected because of their lack of refractility, their ovoid shape, or their pink tinge on staining with 0.1% toluidine blue, then confirmation may be obtained by recognizing budding or, best of all, by an india-ink preparation. Culture presents no problem, and growth of the cryptococcus at 37° is evidence of pathogenicity. Proof of pathogenicity is obtained by intraperitoneal inoculation of white mice or rats. It should be remembered that six months may be necessary for the animals to die and at necropsy the characteristic gelatinous granulomata should be sought and the fungus identified microscopically.

The other components of the cerebrospinal fluid are abnormal, and the biochemical pattern may resemble that found in other meningitides, particularly tuberculous meningitis. Sugar is reduced (Wilson and Duryea, 1951) and may fall as low as 5 mg.%; chlorides are lowered (Wilson and Duryea, 1951; Daniel *et al.*, 1949; Magarey and Denton, 1948; Carton, 1952), while the protein content is raised and the globulin increased (Blair, 1943).

There is usually no dramatic pleocytosis, and this feature mirrors the morbid anatomical finding of remarkably little reaction to cryptococcal parasitism. Such cellular response as occurs is almost entirely lymphocytic. The Lange curve has been reported as abnormal in cases where syphilis has been excluded by other serological tests (Blair, 1943; Wilson and Duryea, 1951; Magarey and

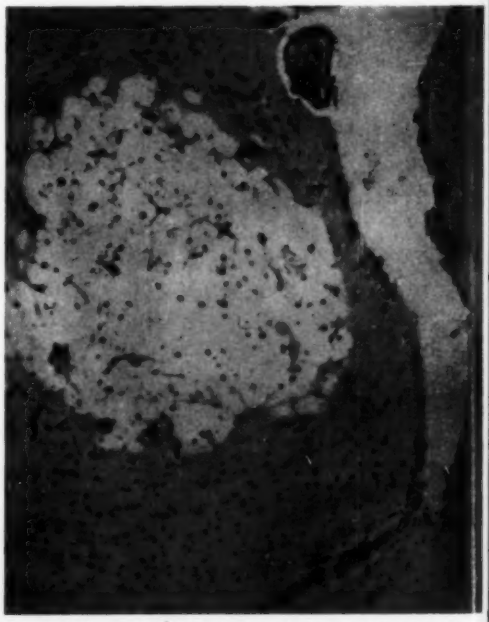

FIG. 6.—Mouse 185/54 brain lesion. Note lack of reaction to the cryptococcal colony. Haematoxylin and eosin, × 100.

Denton, 1948). Therapy may affect the protein and cells, but the sugar content is said by Carton (1952) to be the most sensitive index of the efficiency of treatment.

A comprehensive review has been made of the treatment of cryptococcosis of the central nervous system by Carton (1952). He reported improvement in two cases out of four, one being apparently cured. He suggests that "actidione" should be used, if possible in conjunction with fever-therapy, as was also suggested by Mosberg and Alvarez-DeChoudens (1951). In a personal communication to the authors Carton (1953) advised "polymyxin" as an adjuvant to therapy. Wilson and Duryea (1951) report one case cured with "actidione." Against the particular strain of *C. neoformans* isolated here "actidione" appeared to be the most active *in vitro*, but it did not appear to act *in vivo*.

There has been much speculation (Gendel, Ende, and Norman, 1950; Symmers, 1953) as to the possible relationship between cryptococcosis and Hodgkin's disease. Of 165 cases of cryptococcosis collected by Gendel, 14 had associated Hodgkin's disease. No evidence of lymphoma was found in this patient and lack of necropsy precluded further search. In view of the possibility of some relationship between the deep mycoses and the lymphomata, the former should be excluded as far as possible whenever the latter are diagnosed.

Most textbooks describe the cryptococcus as showing single budding, but several of the organisms found in this case possessed double buds; this was noted also by Emmons (1951). Multiple budding *Blastomyces brasiliensis* is excluded by the large capsule, by the absence of a mycelial phase on culture at room temperature, by the gross features of the colony, and by the histological appearances in inoculated mice.

Summary

A case of cryptococcal meningitis is presented.

The mycological and cerebrospinal fluid findings, as well as the results of animal inoculation experiments, are discussed.

Methods of diagnosis, including the potentialities of 0.1% toluidine blue as a differential stain, are described.

Anti-fungal agents are examined.

We should like to thank Dr. T. Rowland Hill, under whose care the patient was admitted, for clinical details; Dr. R. W. Riddell, of the Brompton Hospital and of St. John's Hospital for Diseases of the Skin, London, and Dr. Jan Schwarz, of the University of Cincinnatti, Ohio, U.S.A., for their confirmation of the identity of the organism; Mr. E. A. Yallop, F.I.M.L.T., Mr. R. M. M. Gould, and Mr. D. Baker for technical assistance; and Mr. J. Wood, F.R.S.A., for assistance with the photography. Actidione was provided through the courtesy of the Upjohn Company, of Kalamazoo, Mich., U.S.A.

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NEEDLE NECROPSY

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The importance of post-mortem examination is generally accepted, not only for instruction and research, but also for raising the standard of medical practice. Unfortunately, there are many obstacles. In hospital, necropsies are limited by the natural reluctance of many relatives to give the necessary permission. In nursing homes and private houses, they are seldom performed because of the additional problems of inadequate facilities and lack of time. Correspondence in the *British Medical Journal* expressed concern at the difficulties of obtaining necropsies on patients dying outside hospital (Loewy, 1953; Davies, 1953; Wade, 1953).

It is, however, possible to obtain adequate diagnostic post-mortem specimens with a needle. Such a procedure does not dismay relatives and can be carried out quickly and without trouble with an appropriate instrument. The purpose of this communication is to discuss the technique of needle necropsy and to describe a suitable instrument which has given satisfactory results during the past five years.

Necropsy Instrument

The instrument is essentially that previously described for needle biopsy of the liver (Terry, 1949) but with a modified needle of larger bore. It consists of a 20 ml. syringe adapted to carry a 3 mm. (internal diameter) needle, 15 cm. long, on the barrel and a trocar on the piston (Fig. 1).

The point of the needle is notched and sharpened to ensure that it cuts into the tissue. A bevelled point has been discarded, since it is blunted by hard tissue such as bone, and fails to obtain specimens from spongy tissue such as lung. The trocar has a cutting point, which projects 1 cm. beyond the needle: it is flattened along one side so that pressures within the syringe, positive or negative, are transmitted down the needle to the specimen. A spring-catch on the cap of the syringe engages with notches on the piston-rod and maintains the piston in place after suction has been applied.

Technique of Needle Necropsy

Needle necropsy can be performed at the bedside as easily as in the mortuary. A pair of rubber gloves is useful but not essential. Ascitic or pleural fluid will escape through the needle track: suture material and cotton-wool should therefore be available to protect the bed and coverings. Several labelled test-tubes containing fixative are necessary for the specimens. The danger of personal injury with the needle, especially in cases of infection, must be constantly borne in mind. No other precautions or preparations are necessary.

The instrument is assembled so that the flattened side of the trocar is adjacent to the notch in the point of the needle: if it is adjacent to one of the two points of the needle, that point will catch in the tissues. The trocar and needle are then inserted through the skin and intervening tissues until the particular organ or tumour is reached. The specimen is obtained by a movement exactly opposite to that used in aspirating fluid with a syringe: the left hand holds the plunger stationary, while the right hand thrusts the syringe barrel forwards towards the cadaver, and so advances the needle into the tissue. The specimen is now within the needle and held by the negative pressure in the syringe, but it is still attached at its distal end. This attachment is broken by further withdrawing the plunger to stretch the specimen and then rotating the whole instrument several times. On removing the instrument from the cadaver, the specimen remains in the distal portion of the needle, and is prevented by the trocar from being sucked up into the syringe.

The specimen is best handled by delivering it into a tapered glass tube. After unscrewing the needle from the syringe the point is inserted into a suitable glass tube and the specimen extruded with an obturator. The specimen is kept straighter if the needle is pulled back over the obturator rather than if the obturator is simply pushed through the needle. The glass tube with its specimen is placed in 10% formol-saline or other fixative in a test-tube. Specimens from different sites are placed in separate labelled test-tubes (Fig. 2).

Applications

Needle necropsy is naturally not of equal value in all cases. Diagnosis is easy with a large nodular liver, whereas it is often impossible in many conditions, such as generalized infections, peptic ulceration and its complications, intestinal perforation and obstruction, vascular disasters, and some cardiac conditions. In general terms, needle

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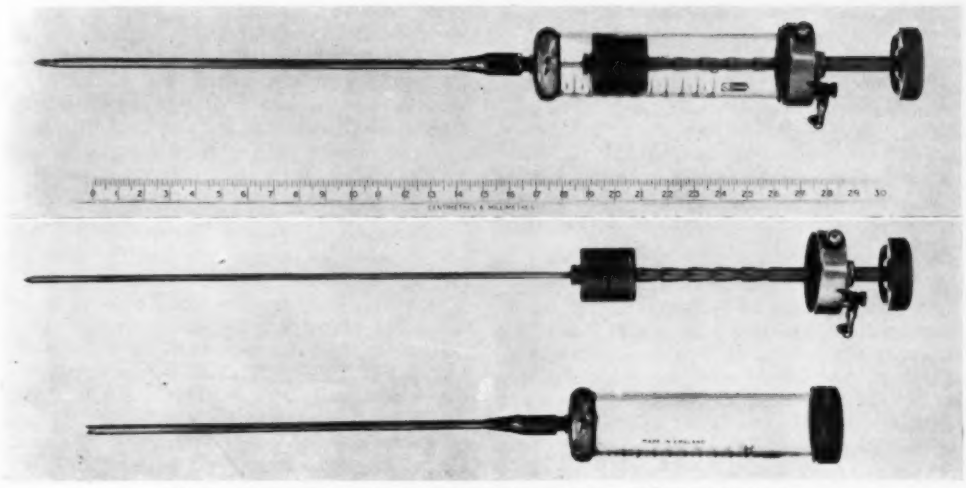

FIG. 1.—Instrument for needle necropsy ready to use (above) and dismantled (below).

necropsy is most likely to be successful when there is a known mass or enlarged viscus.

The brain may sometimes be approached through the foramen magnum, the orbit, or the foramen ovale; it is, however, much simpler to drill a small hole in the skull directly over the suspected area. Meningitis, encephalitis, cerebral tumours, and sometimes vascular disease may be diagnosed.

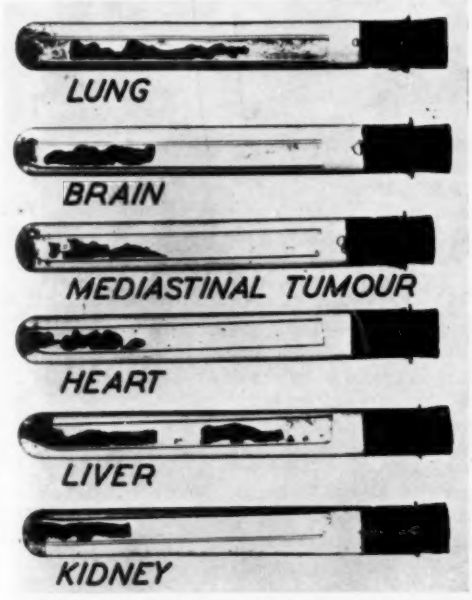

FIG. 2.—Specimens obtained by needle necropsy, each in tapered glass tubes in separate labelled test-tubes containing 10% formol-saline.

The thorax is easily accessible through the intercostal spaces. Specimens from the lungs may reveal pneumonia, phthisis, silicosis, and bronchial carcinoma, while the nature of mediastinal masses and sometimes of cardiac disease may be ascertained.

The abdomen is a rich field for needle necropsy with the liver yielding particularly good results. Hepatic specimens may disclose hepatitis, obstructive jaundice, cholangitis, fatty infiltration, cirrhosis, primary or secondary carcinoma, amyloid disease, tuberculosis, sarcoidosis, brucellosis, histoplasmosis, leishmaniasis, schistosomiasis, hydatid disease, amoebic abscess, Gaucher's disease, haemochromatosis, and polycystic disease. Needle necropsy should probably always include a specimen from the liver, whether or not clinically involved: if not enlarged, it is readily approached through the lower intercostal spaces. The spleen is less informative, except where there are deposits of lymphosarcoma, Hodgkin's disease, or other reticulosis. The kidneys are best approached through the posterior renal angles unless palpable. Renal specimens may reveal nephritis, polycystic disease, tuberculosis, and tumours. Carcinoma may be demonstrated anywhere in the intestinal tract, the pancreas, the suprarenals, the gall bladder, the urinary bladder, and in the pelvis.

Retroperitoneal and pelvic masses may be elucidated.

Other informative sites are enlarged lymph glands, bony swellings, the vertebral bodies, and lumps in the breast.

These possible diagnoses and methods of approach are given only as guides. Individual cases will disclose many other anatomical diagnoses, while the ingenuity of the prosector will suggest other approaches suitable to the particular problem.

Personal Results

In a personal series of 24 needle necropsies, the following diagnoses have been made: Metastatic carcinoma of the liver (7), Laennec's cirrhosis (6), fatty infiltration of the liver (5), bronchial carcinoma (3) and suppurative cholangitis with secondary perisplenic abscess (1). No diagnosis was possible in two cases.

The interpretation of the specimens may require some detective ability as well as skill in histological interpretation. In one instance, the prominent features during life had been jaundice and splenomegaly. Needle necropsy revealed suppurative cholangitis, splenic tissue, and an abscess wall in separate specimens. From the information on the labelled specimen relating to site and method of approach, it was clear that the splenomegaly was in fact a normal spleen displaced by an abscess, and it was possible to make a diagnosis of cholelithiasis with suppurative cholangitis and secondary perisplenic abscess. The diagnosis was confirmed later, when permission for a full necropsy was unexpectedly granted. This case also underlines the importance of accurate and detailed labelling of the specimens.

Discussion

Most needles will, in theory, cut into tissue and obtain specimens, but in practice they are seldom successful, since they fail to detach the core at its base. Numerous instruments have therefore been devised for needle biopsy. During this investigation two of the most popular biopsy needles, the Iversen–Roholm needle (Iversen and Roholm, 1939) and the Vim–Silverman needle (Silverman, 1938) were studied, but they were considered unsatisfactory, since the specimens were frequently damaged and difficult to preserve in a condition acceptable to the pathologist, and, with the Vim–Silverman needle, too small.

The suggested instrument (Fig. 1) is simple to use and provides adequate material for diagnosis. The technique permits the specimens to be placed in glass tubes, where they are preserved straight and intact and are thus easier for the technician to prepare and the pathologist to examine. Once in the glass tube, the specimen can be examined macroscopically for the presence of carcinoma and occasionally other pathological changes. By using a glass tube tapered at one end, not only is the specimen prevented from slipping out of the tube, but the pathologist knows that the tapered end contains the deeper portion of the specimen.

Advantages of Needle Necropsy.—The chief advantage of this procedure is that it permits post-mortem diagnosis when it would otherwise be impossible. Relatives who are unwilling to permit a full necropsy will often consent to needle necropsy. Relatives who permit only limited necropsy will often agree to needle necropsy of the other organs. If a patient dies out of hospital, it is difficult to arrange for a pathologist to come to the nursing home or private house, where the lack of facilities would in any case probably deter him from carrying out a necropsy. The problem and expense of removing a cadaver to a hospital are usually prohibitive. The general practitioner is seldom able to spare the time to perform such an unwonted task in a private house. Needle necropsy, on the other hand, permits the medical attendant to obtain diagnostic specimens in a few moments at the bedside.

Needle necropsy is also valuable when it is desired to obtain some particular tissue as soon after death as possible, even though a post-mortem permit has been signed. The post-mortem examination may be delayed for several days owing to pressure of work or statutory holidays; or the tissue, e.g., the liver, concerned may be liable to rapid post-mortem autolysis. Under these circumstances, early needle necropsy will provide better histological specimens than those obtained at the later formal examination.

The idea of obtaining post-mortem specimens by some form of needle or instrument is not new. Manson-Bahr (1950) refers to the value of the viscerotome in South America during the investigations of yellow fever and leishmaniasis in regions where post-mortem examinations were not permitted by the natives. Post-mortem specimens are obtained by biopsy needle in some institutions where needle biopsy is practised. However, the concept of needle necropsy remains little known, and the needles available are ill-suited for the purpose. It has therefore seemed worth while to draw attention to this procedure and to its advantages, and to describe an instrument which has given the best results during a period of five years' experimental and practical use.

Summary

Needle necropsy is a valuable alternative to formal post-mortem examination, not only in private houses and nursing homes, but also in hospital.

An instrument suitable for needle necropsy is described and its use discussed.

My thanks are due to Mr. W. T. Harris, of W. H. Bailey and Sons, for his assistance in producing the instrument for needle necropsy, and to the Photographic Department, St. Bartholomew's Hospital, for the illustrations.

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J. clin. Path. (1955), **8**, 42.

THE SPLEEN AND BLOOD EOSINOPENIA

BY

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Within a few hours of injury or burning the number of eosinophils in the blood falls progressively to reach low or zero values and this eosinopenia (Fig. 1) lasts up to a few days (Laragh and Almy, 1948; Roche, Thorn, and Hills, 1950; Hardy, 1950; Sevitt, 1951, 1954; Evans and Butterfield, 1951).

The endocrine control of eosinopenia is known to be due to release of adrenocortical hormones through stimulation of the anterior pituitary, but the manner in which the eosinopenia is produced is in doubt. The question has been recently reviewed by Essellier, Jeanneret, and Morandi (1954). Several possible explanations could account for the eosinopenia, viz., (1) inhibited release of eosinophil leucocytes from the bone marrow, (2) destruction of eosinophils in the blood, (3) redistribution of the eosinophils in the tissues, and (4) redistribution followed by destruction in the tissues.

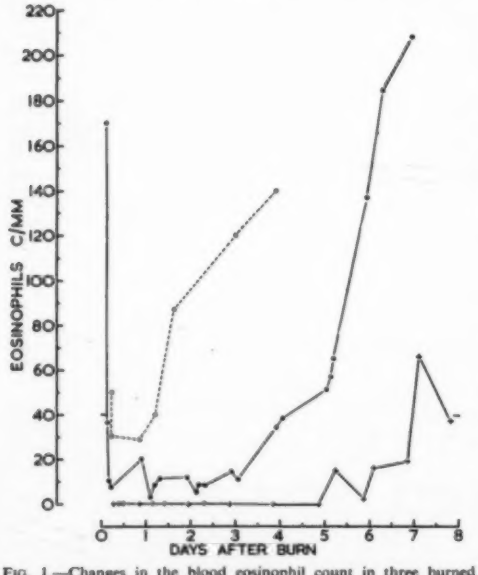


FIG. 1.—Changes in the blood eosinophil count in three burned patients. In one patient the falling of the count soon after burning is seen and this is followed by a period of eosinopenia which is present in all three. The eosinopenia lasted one, five, and seven days respectively and was followed by a rise in the count.

In this paper the role played by the spleen in redistribution and destruction will be considered.

Eosinophils in the Normal Spleen

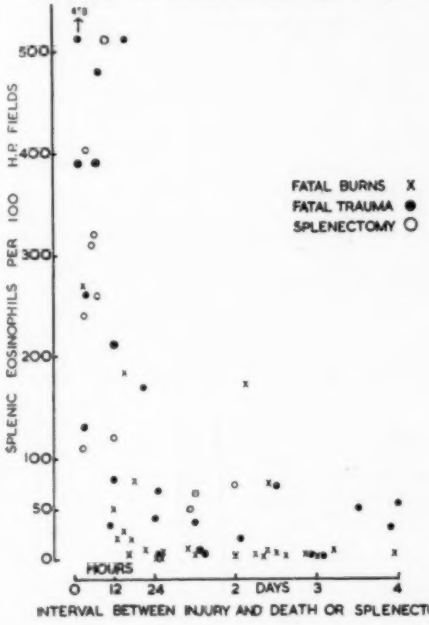
The abundance of eosinophils in the spleens of previously healthy subjects dying within a few hours of trauma or other catastrophe and of those splenectomized within a similar period indicates that the normal spleen contains many eosinophils (Sevitt, 1955). Similar numbers of eosinophils in the spleens of patients with Addison's disease indicate that splenic eosinophilia is not due to a rapid post-traumatic filtration from the blood under the influence of adrenocortical hormones. The densities of the eosinophils in 5 μ histological sections usually range from 200 to 700 per 100 high-power fields or from 6,000 to 25,000 per c.mm. of tissue. This is many times the normal concentration in the blood, and since the normal spleen is not eosinopoietic (Biggart, 1932) the spleen must normally filter eosinophils from the blood.

Splenic Eosinophils after Burning and Trauma

It was also found that within 12 to 24 hours of severe injury or burning the number of eosinophils in the spleen becomes reduced until eosinopenic values of fewer than 50 eosinophils per 100 high-power fields are reached (Fig. 2). Indeed, nearly every patient dying one to several days after injury or burning died with an eosinopenic spleen.

In a number of patients dying between 12 and 24 hours after injury or burning, splenic eosinophilia was found. At this time, however, the blood eosinopenia would have been well established. This fact suggests that eosinopenia of the blood precedes eosinopenia of the spleen. Further evidence of this hypothesis was found by following the blood eosinophil counts after injury in three

FIG. 2.—Splenic eosinophil counts (per 100 high-power fields) in three groups of patients, fatally burned, fatally injured, and splenectomized. These are related to the intervals between injury and death or splenectomy. During the first 24 hours many of the spleens contain many eosinophils varying from 100 to 658 per 100 high-power fields. After this nearly all the spleens contain relatively few eosinophils, particularly in the patients dying from burns. These results indicate that splenic eosinopenia occurs within 12 to 24 hours of injury or burning and continues (or is renewed) during the next few days.



patients later undergoing splenectomy for traumatic rupture of the spleen, and then estimating the densities of the eosinophils in the spleens.

The blood eosinophil counts two, two and a half, and 13 hours after injury were only 6, 3, and 1 per c.mm. (Fig. 3), whereas the spleens removed one, four, and two hours later contained considerable numbers of eosinophils (238, 322, and 507 per 100 high-power fields). That these spleens would have become eosinopenic some hours later had they not been removed is shown by relating the eosinophil values in 11 surgically removed ruptured spleens (including the spleens of these three patients) to the intervals between the accident and splenectomies. The five spleens removed within three to seven hours of injury were eosinophilic (110 to 403 eosinophils per 100 high-power fields), the spleens removed 12 and 13 hours after injury also had densities of 118 and 507 eosinophils, while lower values of 50, 64, and 74 respectively were found in the organs removed 36, 36, and 48 hours after injury.

These findings are consistent with two hypotheses. First, the spleen removes eosinophils from the circulation and destroys them. Secondly, a non-splenic mechanism which produces blood eosinopenia also produces eosinopenia of the spleen through the discharge of splenic eosinophils into the blood stream.

Necrotic and Degenerate Eosinophils in the Spleen after Injury and Burning

Necrotic eosinophils had ill-defined karyolytic nuclei or were cells without nuclei. In the latter only the bright red granules were enclosed in a cytoplasmic membrane, and sometimes when the membrane was ruptured the eosinophils appeared merely as an irregular clump of free granules. These eosinophils were not included when estimating the eosinophil leucocyte densities. Other changes affected the granules, which were sometimes swollen and appeared to coalesce or were reduced in density.

More necrotic eosinophils were found in spleens containing many normal eosinophils than in those containing few of these cells. The former usually contained 20 to 70 necrotic cells per 100 high-power fields ; for example, the spleen of a man dying three hours after burning had 37 necrotic eosinophils and 273 normal eosinophils per 100 high-power fields. On the other hand, the density of necrotic eosinophils in the eosinopenic spleens was small, varying usually from 1 to 8 per

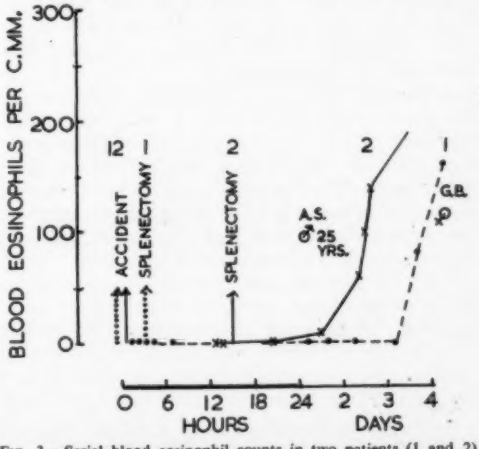


FIG. 3.—Serial blood eosinophil counts in two patients (1 and 2) before and after splenectomy for traumatic rupture of the spleen. Blood eosinopenia was established before operation and continued after splenectomy for about two and a half and one and a half days. Both spleens were eosinophilic (238 and 507 eosinophils per 100 high-power fields), indicating that blood eosinopenia occurs before splenic eosinopenia.

100 high-power fields ; for example, the spleen of a patient dying three days after severe burning had 1 necrotic and 2 normal eosinophils per 100 high-power fields.

The presence of these necrotic and degenerate cells suggests that the spleen either destroys viable eosinophils or removes degenerate eosinophils from the circulation.

Eosinopenia in Patients Splenectomized after Trauma

Eight previously healthy males, including four whose spleens were sectioned, varying in age from 14 to 61 years, were studied. Each had received injuries to the thorax and/or abdomen resulting in a rupture of the spleen. The patients were transfused with blood. The spleens of six were removed under general anaesthesia three to seven hours after injury, in the seventh at 13 hours, and in the eighth operation was delayed until 48 hours because of difficulty in diagnosis.

Spontaneous Eosinopenia.—Blood eosinophil counts, two to four times a day for three to five days, were carried out on seven patients. In four, the counts were started three to 12 hours after splenectomy, and in the three others one, two, and four hours before operation. A profound eosinopenia was found in each patient with very low or zero eosinophil counts, and this lasted one and a half to two and a half days (Fig. 3). The eosinopenia was ended by a sudden and progressive rise in the count. In five patients the blood eosinophil counts returned to normal values in two or three days ; in the other two temporary eosinophilic values were found (maximum counts 1,200 and 788 per c.mm.).

Similar degrees and periods of eosinopenia are found after severe trauma and major abdominal operations (Laragh and Almy, 1948 ; Roche *et al.*, 1950 ; Hardy, Richardson, and Dohan, 1953 ; Sevitt, unpublished observations). The absence of the spleen, therefore, did not appear to influence the degree of duration of the eosinopenia due to post-traumatic adrenocortical activity. Such activity is intense and probably maximal, and the absence of the spleen may have been compensated for by other mechanisms. The spleen's role in the production of eosinopenia might be disclosed by submaximal stimulation of the adrenal by A.C.T.H.

Induction of Eosinopenia by A.C.T.H.—Among the effects of A.C.T.H. through its stimulation of the adrenal cortex is a considerable reduction in the blood eosinophil count, and this eosinopenic property has been used as a method of evaluating adrenocortical function (Thorn, Prunty, and Forsham, 1947 ; Dougherty and White, 1947 ; Hills, Forsham, and Finch, 1948 ; Forsham, Thorn, Prunty, and Hills, 1948 ; Sevitt, 1954). Injecting 20 to 25 mg. of A.C.T.H. lowers the eosinophil count usually within an hour of injection and always within two hours (Hills *et al.*, 1948 ; Sevitt, 1954) ; the maximum fall (Fig. 4) in the count occurs three to five hours, usually four hours, after injection and nearly always ranges between 60% and 90% of the pre-injection count (mean value 70%–75%).

Seven of the splenectomized patients were tested with A.C.T.H. when the spontaneous blood eosinopenia had ended and either during or shortly after the post-eosinopenic rise of the eosinophil count. The patients were well and there was no evidence of pituitary or adrenocortical dysfunction. Therefore, any abnormality in the response of the

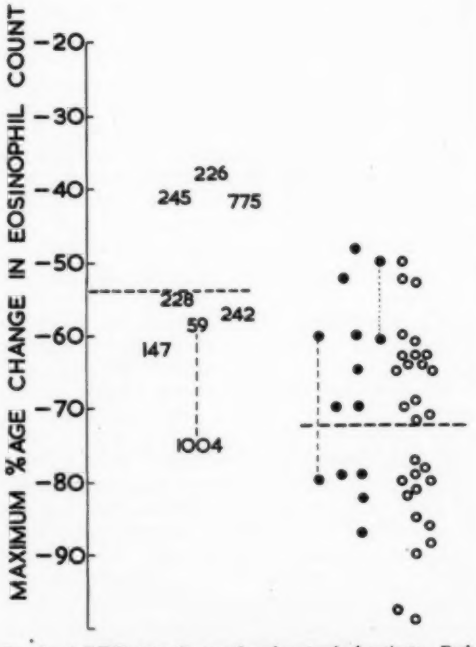


FIG. 4.—A.C.T.H. tests in recently splenectomized patients. Each point is the result of one test and is represented by a numeral which is the pre-injection value of the blood eosinophil count per c.mm. Its vertical position expresses the maximum percentage lowering of the count. To compare with these are the results of A.C.T.H. tests in burned and other patients. The horizontal interrupted lines are the mean values in the two groups. The vertical lines join the results of tests on one patient. Three of the tests on the splenectomized subjects were definitely abnormal and the values are significantly less than the values obtained in the burned patients (see text). ● Burned patients. ○ Other patients.

blood eosinophils to the injection of A.C.T.H. would be evidence, not of adrenocortical dysfunction but of the role which the spleen plays in the production of blood eosinopenia.

Methods

The A.C.T.H. (batches J16205, J27511, and J28112) used was previously found to produce the expected eosinopenia in normal and burned persons. One intramuscular injection of 20 mg. freshly dissolved in about 2 ml. of distilled water or saline was given between 11 a.m. and 1 p.m.

Blood eosinophil counts on capillary blood were made by the direct "wet field" method of Dunger (1910) using a modified eosin-acetone water diluent. The blood was diluted to 1 in 20 in each of two pipettes and the number of eosinophils in four Fuchs-Rosenthal fields, corresponding to 0.65 c.mm. of blood, were counted.

Counts were made 15 to 60 minutes before the injection, at the time of injection, and hourly thereafter for four to six hours. The eosinophil counts were plotted, the shape of the curve was examined, and the maximum fall of the count was expressed as a percentage of the pre-injection count.

Results

Eight A.C.T.H. tests were carried out on seven patients. Tests on six patients were made on the third, sixth, sixth, seventh, eleventh, and thirteenth days after splenectomy, and on the fourth and eleventh days in the seventh patient.

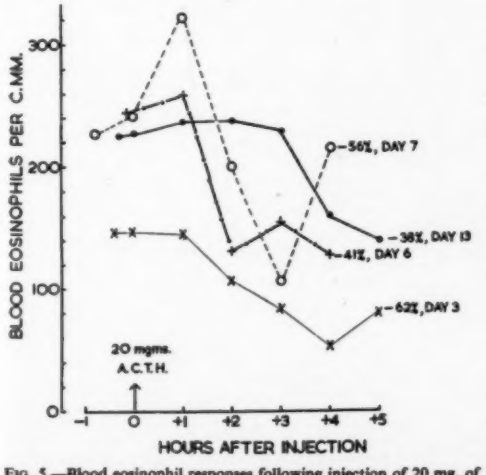


FIG. 5.—Blood eosinophil responses following injection of 20 mg. of A.C.T.H. to recently splenectomized patients. Only one of the four curves (X—X) is normal. In one patient the count rises considerably at one hour after injection and then falls; in another there is an abnormal delay of three hours before the count falls; in two patients the counts fall by only 38% and 41% of the pre-injection values.

Eosinopenia was induced in each patient, but the results from only two subjects fulfilled the minimum criteria of normality. In two patients (three tests) the counts did not fall for the first two or three hours after injection; indeed, increases of 5% to 20% over the pre-injection levels developed. In two others the counts increased by 5% and 33% respectively at one hour after injection but at two hours had fallen. This pre-eosinopenic increase of the eosinophils lasting one to three hours is not seen in normal persons or in burned patients.

Although the lowest eosinophil counts occurred at the normal time, three to five hours after injection of A.C.T.H., often they were less than normal. In three patients the counts fell by only 38%, 41%, and 43% of the pre-injection levels, in the other four subjects (five tests) the counts fell by 55%, 57%, 62%, 59%, and 75%. The mean percentage decrease in the eight tests was 53% compared to a mean fall of 70% to 75% in normal persons and 67% in burned patients (Fig. 4). Statistical analysis by the t test showed a significant difference between the results in the splenectomized and burned patients ($p<0.05$).

Discussion

Eosinopenia can be induced in splenectomized rats and mice by adrenaline and A.C.T.H. (Speirs and Meyer, 1949; McDermott, Fry, Brobeck, and Long, 1950) so that the spleen is not essential for eosinopenia in these animals, but whether it plays some part in the intact animal is in dispute. Spain and Thalhimer (1951) claimed that eosinophils temporarily accumulate in the spleens of mice after cortisone, but no histological increase was observed by Mach, Brügger, Della Santa, and Fabre (1950). Moreover, Solomon and Humphreys (1951) found that during the development of blood eosinopenia in dogs the splenic veins often contained more eosinophils than the femoral arteries. This would indicate that the spleen discharged eosinophils into the circulation while eosinopenia of the blood is taking place.

It has now been found that blood eosinopenia lasting one and a half to two and a half days occurs or continues spontaneously after splenectomy. Soon after this it can be induced by A.C.T.H. Therefore the spleen is not essential for eosinopenia in man.

A similar conclusion was reached by Essellier, Morandi, and Stein, who found that a similar type of eosinopenia was induced by A.C.T.H. in both normal and splenectomized subjects. However,

this has not been the present experience. The eosinopenia induced by A.C.T.H. may be preceded by a temporary increase in the eosinophil count and/or prolonged delay of one to three hours before the count falls. This indicates that after A.C.T.H. eosinophils are mobilized into the blood from other body tissues, and suggests that this early mobilization is normally masked by the spleen. The degree of eosinopenia induced by A.C.T.H., often less than is normal, also suggests that the spleen plays a part in the mechanism of blood eosinopenia. The persistence of splenic eosinophilia for 12 to 24 hours after trauma when blood eosinopenia has already occurred, and the presence of necrotic eosinophils in most spleens, also suggests that the spleen filters eosinophils from the blood and destroys them. Essellier and Wagner (1952) found that blocking the reticulo-endothelial system in guinea-pig by tryptan blue prevented the induction of eosinopenia by A.C.T.H. If this were true for man, splenectomy would be expected to produce a variable effect upon the rate and degree of eosinopenia induced by A.C.T.H., depending upon the proportion of the reticulo-endothelial system contained in a particular spleen. Such a variability, which occurred in the splenectomized patients after A.C.T.H., supports the belief that the reticulo-endothelial system, including the spleen, is responsible for the removal and destruction of the eosinophils from the blood.

The role of the spleen may be a minor one in some patients and hidden by other mechanisms: in other subjects it is more important, but the spleen is not essential for the production of blood eosinopenia.

Summary

The spleen is not essential for the production of blood eosinopenia in man, since this occurs spontaneously after splenectomy and can be induced by A.C.T.H. in recently splenectomized patients. However, the eosinopenia induced is frequently less than occurs in normal subjects and may be preceded by an abnormal delay before the eosinophil count falls, during which period it may rise. Moreover, the blood eosinopenia after injury precedes eosinopenia of the spleen and this organ contains necrotic as well as normal eosinophils. It is therefore suggested that the spleen plays some part in the removal and destruction of the eosinophils from the blood and in the production of eosinopenia.

I am grateful to my surgical colleagues, particularly Mr. R. Clarke and Mr. J. S. Horn, for their helpful co-operation. My thanks are also due to Miss A. J. Fisher, B.Sc., for statistical help, to Mr. C. Richardson, of the Photographic Department of the Accident Hospital, and to my secretary, Miss M. Hayes.

The work was carried out in my capacity as a part-time member of the scientific staff of the Medical Research Council.

The A.C.T.H. was obtained through the courtesy of the Allocations Committee of the Medical Research Council and was prepared by Armour Laboratories.

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J. clin. Path. (1955), **8**, 47.

THE USE OF A NEW CETRIMIDE PRODUCT IN A SELECTIVE MEDIUM FOR *PSEUDOMONAS PYOCYANEA*

BY

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(RECEIVED FOR PUBLICATION MAY 7, 1954)

A selective medium containing 0.1% cetrimide in nutrient agar has been used here during the past four years for the routine identification of *Ps. pyocyanea* in burns (Lowbury, 1951). This medium, a modification of those previously described by Harper and Cawston (1945) and by Hood (1948), has proved convenient and efficient.

In October, 1953, the manufacturers announced the production of a modified "cetavlon" which was claimed to be more active against *Ps. pyocyanea* than the old product, and little less active against that organism than against *Proteus vulgaris*. In view of these observations we have made the following tests to find whether the new cetrimide is suitable for use in a selective medium for *Ps. pyocyanea*, and if so at what concentration it should be included.

Choice of a Dilution of the New Cetrimide for the Selective Medium

Media containing the old and the new cetrimide were compared by methods described by one of us in a previous paper (Lowbury, 1951).

The basic medium was "lemco" agar at *p*H 7.8 ("oxoid lab. lemco," 1.0 g., "oxoid" peptone, 1.0 g., sodium chloride, 0.5 g., Davis agar, 1.5 g., and tap water 100 ml.). The quality of growth, pigment, and fluorescence of *Ps. pyocyanea* was somewhat improved by the addition of a phosphate buffer to this medium. A series of dilutions of the new cetrimide, from 0.01% to 0.1%, was incorporated in the basic medium, and these were compared with the old cetrimide agar (0.1%) in respect of (*a*) ability to inhibit the growth of *Proteus vulgaris*, coliform bacilli, and some other organisms, (*b*) the growth of large and small inocula of *Ps. pyocyanea*, and (*c*) the isolation of *Ps. pyocyanea* from burns.

TABLE

EXAMINATION OF 500 BURN SWABS WITH OLD AND NEW CETRIMIDE AGAR

Medium*	*Ps. pyocyanea* Isolated on					
	Direct Plating of Swabs			Subculture from Fluid Medium		
	Positive at 18 hrs.	Positive at 48 hrs.	Total +	Positive at 18 hrs.	Positive at 48 hrs.	Total +
Agar with new cetrimide (0·03%) ..	116	50	166	230	13	243
Agar with old cetrimide (0·1%) ..	94	63	157	227	15	242

* The order of inoculating these media was reversed with successive swabs to make the two series comparable.

The lowest concentration of new cetrimide sufficiently selective for use in the medium was found to be 0.03%. A trial of this medium on swabs taken from burns (see Table) showed that it was at least as effective in showing the presence of *Ps. pyocyanea* as the old medium. *Ps. pyocyanea* was recognized by the production of a green pigment, or of fluorescence under ultra-violet irradiation, or of both (Lowbury, 1951). Colonies of *Ps. pyocyanea* were larger on the new medium than on the old, and the proportion of isolations obtained after overnight incubation was also greater with the new medium. With cetrimide at a concentration of 0.04%, however, the growth of *Ps. pyocyanea* from swabs was poor, and small inocula often failed to grow. From these observations we inferred that the optimal concentration of the new cetrimide was 0.03%.

Comments

Ringen and Drake (1952) recently described the use of pyocyanin in a fluid selective medium for *Ps. pyocyanea*. We have made tests with agar media containing up to 0.05% pyocyanin, and found that they were no more selective for *Ps. pyocyanea* than cetrimide agar. They were, moreover, unsatisfactory in use because the characteristic pigment by which *Ps. pyocyanea* is identified could not be seen.

Occasionally *Ps. pyocyanea* on cetrimide agar has been found to lose its fluorescence under ultraviolet irradiation after standing for a short time on the bench. It reappeared, however, when the plates were reincubated.

As cetrimide, even in its new form, is not a pure compound, we recommend that new batches should be tested to establish the optimal concentration required in the selective medium for *Ps. pyocyanea*.

Summary

A trial on burn swabs has shown that the optimal concentration of a new cetrimide product in a selective medium for *Ps. pyocyanea* is 0.03%. Growth, fluorescence, and pigment production on this medium could be improved by inclusion of a phosphate buffer at *p*H 7.8.

We wish to thank Imperial Chemical (Pharmaceuticals), Ltd., for information about the new "cetavlon," and Dr. A. C. Cunliffe for strains of *Proteus vulgaris*.

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THE OCCURRENCE OF *PASTEURELLA SEPTICA* (syn. *MULTOCIDA*) IN BRONCHIECTASIS

BY

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(RECEIVED FOR PUBLICATION JULY 28, 1954)

There are a number of British reports of the isolation of *Past. septica* from wounds (Allott, Cruickshank, Cyrlas-Williams, Glass, Meyer, Straker, and Tee, 1944; Cooper and Moore, 1945; Brunsdon and Mallett, 1953) and from other situations in the human body, such as the appendix (Ludlam, 1944) and joint spaces (Pizey, 1953), but with the exception of the case of sinusitis reported by Bartley and Hunter (1947) the occurrence of this organism in the respiratory tract has not previously been described in this country.

In the following case *Past. septica* was repeatedly isolated from the sputum of a patient with bronchiectasis over a period of several months.

Case Report

C. B., a watchmaker, aged 29, had a history of recurrent attacks of bronchitis and asthma since the age of 10 years. These did not cause him much incapacity until 1950, when he was admitted to hospital with a severe attack of bronchitis; since then he has been cyanosed and has had a decreased exercise tolerance. In February, 1953, he was admitted to the Brook Hospital, London, with purulent bronchitis and finger clubbing, and was suspected of having incipient cor pulmonale. His sputum was examined bacteriologically on March 9, a mixed bacterial flora with no predominant organism being found. He was discharged from this hospital much improved after six weeks' treatment.

In July, 1953, his condition deteriorated and he was admitted to King's College Hospital dyspnoeic and persistently cyanosed at rest. He was producing a considerable quantity of purulent sputum. A report at this time stated that bronchograms of the right side showed slight dilatation of the upper lobe branches with mild fusiform bronchiectasis, and diminution in volume of the middle lobe with slight dilatation of the dorsal mid-basic and cardiac branches of the lower lobe. On the left side fusiform dilatation of a moderate degree affecting the lingular, middle, and posterior basic segments of the lower lobe was seen. The results of physical examination, of electrocardiograms, and of cardiac catheterization were compatible with a diagnosis of right heart cardiac hypertrophy due to pulmonary hypertension.

Cultures of his sputum on July 3 and July 10 produced a heavy growth of *Past. septica*.

A course of aureomycin (2 g. daily for six days) was given, at the end of which *Past. septica* was no longer recovered from the sputum, which yielded a heavy growth of *Proteus vulgaris*; with breathing exercises and postural drainage the patient's condition further improved, and he was discharged on July 22 after three weeks in hospital.

Sputum obtained one month after discharge grew only *Proteus vulgaris*, but *Past. septica* was isolated on October 10 from a specimen which yielded a predominant growth of *Proteus vulgaris*. Sputum examined on November 9 yielded a predominant growth of *Past. septica*; *Proteus vulgaris* was not isolated. Though at this time the patient's general condition had deteriorated considerably, there was no reason to believe this was attributable to infection with *Past. septica*.

As it is suggested that animals are the source of infection with this organism in man, it should be recorded that, since disposing of his dog in 1949, the patient had had no close contact with animals.

Bacteriology

In smears from the initial aerobic culture the pasteurella was seen as a small Gram-negative coccobacillus measuring approximately 1.5 μ × 0.5 μ. Bipolar staining was not observed. In cultures on 5% horse-blood agar plates capsules were readily demonstrated. There was no observed motility either at 37° C. or at 22° C.

Cultural Characteristics.—On horse blood agar after 18 hours' aerobic incubation at 37° C. there was a profuse growth of round, grey, convex, translucent shiny colonies, 1–2 mm. in diameter. There was no haemolysis.

Growth in nutrient broth produced a uniform turbidity and a slight powdery deposit after 18 hours. A gelatin stab showed a filiform growth but no liquefaction after a week. There was no growth on MacConkey's agar after a week.

Biochemical Reactions.—Acid, but no gas, was produced in glucose, sucrose, mannitol, galactose, sorbitol, and xylose, and weakly in maltose. There was no fermentation in adonitol, arabinose, dulcitol, glycerol, glycogen, inositol, inulin, lactose, raffinose, rhamnose, or trehalose.

The other biochemical tests were as follows :

Litmus milk: no change.
Methylene blue reduction: positive.
Methyl red: negative.
Voges Proskauer: negative.
H_2S production: negative.
Indole: positive.
Catalase: strongly positive.
Nitratase: strongly positive.
Citrate utilization: negative.

Pathogenicity Tests.—A guinea-pig inoculated intraperitoneally with 0.5 ml. of an 18-hour broth culture of the organism died within 18 hours. One pair of albino mice received 0.2 ml. of the undiluted culture intraperitoneally, and another pair 0.2 ml. of a 1 in 100 dilution. After 18 hours, one of the first pairs of mice was dead and the other moribund ; of the second pair, one mouse was moribund and the other obviously sick.

At necropsy, large numbers of short Gram-negative rods showing bipolar staining were seen in smears of the spleen and heart blood of all five animals.

Serology

This strain was not agglutinated by any of three "O" sera (D/5107/51, D/3607/51, D/4307/51) with one of which the majority of animal pasteurellae are agglutinated (Smith, 1953).

A slide agglutination test with the patient's own serum and organism was weakly positive, but tube tests conducted at 37° C. for somatic antigens and 4° C. for capsular antigens showed no agglutination above a titre of 1 in 10.

Sensitivity to Antibiotics

Using a plate technique with a filter paper strip, the organism was found to be sensitive to penicillin, streptomycin, chloramphenicol, aureomycin, and terramycin.

Discussion

Although not reported in this country before 1944, human infection by *Past. septica* is by no means uncommon. The majority of recorded cases have been reviewed by Lévy-Bruhl (1938), Regamey (1939), and Schipper (1947), and fall into two main categories: sepsis following trauma and infections of the respiratory tract. There are a few cases falling into neither class, in which the organism has been recovered from the gastro-intestinal tract, from the meninges, from the pericardial sac, and from infected joints. There is almost invariably a history of direct animal contact in cases of wound infection, the majority of which follow the bites of cats or dogs, and, very occasionally, those of other animals, e.g., rabbit and panther.

In the more deep-seated infections, on the other hand, evidence of animal contact is generally lacking ; for example, it was present in only one of 13 cases reviewed by Schipper. Regarding respiratory tract infection, our knowledge as to direct or indirect animal contact is very limited, since we have no information on this point from the largest group of these cases. These were reported by Needham, in a personal communication to Bezjak and Mimica (1952), who states only that he isolated *Past. septica* from 28 patients "nearly all of whom were suffering from bronchiectasis."

Too much stress, we feel, need not be given to a history of animal contact in chronic cases, because of the well-known ubiquity of *Past. septica* in diseased and healthy animals, such as cats (Schenk, 1938), dogs (Smith, 1953), and rats (Schipper, 1947) ; thus it would appear that few people are able to avoid contact with the organism on some occasion. There is evidence (Smith, 1953) that most pasteurellae isolated from human infection following bites can be recognized serologically as belonging to the types commonly found in the animals concerned, but in at least two cases of human infection without history of animal contact, e.g., our case and one of pericardial infection (Harries, 1954), the strain differed serologically from the usual domestic animal types, a finding which suggests that the source of infection in these cases was different. On the other hand, some modification may have occurred in the strains during residence in the human body.

While there is no doubt as to the pathogenicity of *Past. septica* in the case of traumatic infections of the soft tissues, which often involve also the underlying bone, and the occasional infections of serous surfaces or the meninges, the assessment of the role of this organism when found by chance in the sputum is much more difficult. In the present case, *Past. septica* was found in the sputum on four occasions over a period of several months, most often as the predominant organism ; it was thus obviously no chance contaminant of the respiratory tract but an integral part of the bronchial flora. Like the majority of the cases described by Needham (1948) and those of Mulder (1938) and Bezjak and Mimica, this finding was associated with bronchiectasis.

Since it is proverbially difficult to assess the role of any given organism in bronchiectasis, it is impossible to determine the part played by *Past. septica* in the pathology of these cases ; it

may well be no more than the colonization of an already damaged bronchial tree.

There is little doubt, as Needham suggests, that *Past. septica* is more common in the respiratory tract than is generally supposed, and his isolation of this organism from 11 cases in the Mayo clinic during 1947 bears out this contention. This may partly be due to the fact that the cultural characteristics of *Past. septica* are such that it may easily be overlooked, especially if it is not grown initially in pure culture. Brunsdon and Mallett have suggested that the organism would be isolated more often from wounds of animal origin if routine cultures were taken in casualty departments, and confirmation of this point has been provided by the isolation of *Past. septica* from two wounds of this nature swabbed as part of a survey of staphylococcal finger infections which was undertaken at this hospital from August to November, 1953.

Summary

A case of bronchiectasis is described in which *Past. septica* was isolated from the sputum on several occasions over a period of four months. The source of infection in this and other cases mentioned in the literature and the possible relationship of the organism to the disease is discussed.

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THE FATE OF *S. TYPHI* IN BLOOD CLOT IN RELATION TO THE PROBLEM OF ISOLATION

BY

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Bile salt broth containing streptokinase (100 units/ml.) has in our experience proved superior to ordinary bile broth as a culture medium for the isolation of *S. typhi* from blood clot (Thomas, Watson, and Hewstone, 1954). This superiority refers both to the total number of positive isolations and to the shorter duration of incubation required. In a series of 18 cases (16 typhoid and two paratyphoid A), where one half of each clot was incubated in 15 ml. of streptokinase broth and the other half in 15 ml. of bile broth alone, six of the latter were negative after 14 days' incubation, but the corresponding halves in streptokinase broth produced a growth of *S. typhi*.

In view of the failure of these six half-clots to produce a positive result a study was undertaken of the fate of *S. typhi* in blood clot.

Preparation of Artificially Infected Clots

Serial tenfold dilutions were prepared from an overnight digest broth culture of a freshly isolated strain of *S. typhi* in 0.85% sterile saline. Plate count estimations were performed, and in this way a suspension containing approximately 40 viable organisms per ml. was prepared. One-millilitre volumes were placed in 50 sterile 3 in. by ½ in. tubes and 4 ml. of blood from a donor added to each. The blood-bacterium suspension was well mixed and the blood allowed to clot. After two hours at room temperature the separated serum was removed and each clot bisected using sterile Petri plates and microscope slides as already described (Thomas *et al.*, 1954). One-half of each clot was then incubated in 15 ml. of streptokinase bile broth and the other half in 15 ml. of bile broth in universal containers. Subcultures were plated daily on to Wilson and Blair's medium for 14 days. Results are detailed in Table I.

TABLE I
ISOLATION OF *S. TYPHI* FROM CLOT IN STREPTOKINASE AND BILE BROTHS

Day	Streptokinase Bile Broth		Bile Broth Alone	
	Positive	Negative	Positive	Negative
1	43	7	22	28
2	50	Nil	22	28
3	—	—	24	26
4	—	—	30	20
8	—	—	32	18
9	—	—	34	16
14	—	—	34	16

Table I shows that 16 (32%) of the half-clots in bile broth alone were negative after 14 days. All those in streptokinase broth gave a positive result after two days' incubation. Examination of the culture bottles after 14 days showed that 64% of the bile broth series still contained large volumes of formed clot. In the streptokinase broth series clot lysis was complete in 48 hours in each case. Of the 16 negative cultures all contained formed clot.

At the end of the 14-day incubation period each clot from the 16 negative containers was transferred to a fresh container with 15 ml. of streptokinase broth. Lysis occurred quickly in each case, in 18 to 24 hours, but in spite of this no further positive isolations were obtained when subcultures were plated daily for a further 14 days. The original organisms in fact appeared to have died off. Since the corresponding halves of each of these clots gave positive isolations in streptokinase broth it is not likely that the halves cultured in bile broth for 14 days were initially sterile.

Effect of Storage of Infected Clots at 4° C. and at 37° C.

Twenty-four artificially infected clots were prepared and bisected as described. Each half contained approximately 25 viable organisms. One-half of each was incubated immediately in 15 ml. of streptokinase broth. The remaining half-clots were divided into four groups, two being stored at 4° C. for seven and 14 days respectively and two at 37° C. for similar

periods. Each half-clot was then added to 15 ml. of streptokinase broth and incubated at 37° C. Subcultures were plated daily as before. Results are shown in Table II.

TABLE II

EFFECT OF STORAGE OF INFECTED CLOTS AT 4° C. AND 37° C.

4° C.				37° C.			
Clot No. for 7 Days		Clot No. for 14 Days		Clot No. for 7 Days		Clot No. for 14 Days	
1	+1	7	+1	13	+2	19	−14
2	+1	8	+1	14	+3	20	−14
3	+1	9	+1	15	+4	21	−14
4	+1	10	+1	16	−14	22	−14
5	+1	11	+1	17	−14	23	+2
6	+1	12	+1	18	+4	24	−14

+ = Positive isolation. − = Negative result. Figures after + and − signs = number of days' incubation.

The second series of 24 half-clots resulted in only four positive isolations after subculturing daily for 14 days. The corresponding halves incubated immediately in streptokinase broth all gave a positive result after two days' incubation.

The above results appear to support the contention that viable organisms may be killed off in the clot during the preliminary phase of incubation of either the clot alone or clot in bile broth. The complete lysis occurring in streptokinase broth would liberate organisms trapped in the fibrin meshwork and allow them to proliferate if still viable.

It appeared that one or both of two factors might be responsible for these findings: (1) sufficient natural antibody and complement might be adsorbed on the clot meshwork to produce a bactericidal action; or (2) phagocytic mechanisms in the clot might produce a similar result.

The following methods were adopted to study the problem more fully.

Incubation of S. typhi in Citrated Blood after Removal of Plasma

Five ml. volumes of blood from a donor were added to 1 ml. volumes of *S. typhi* suspension containing approximately 60 viable organisms, and 0.5 ml. volumes of sterile 3.8% sodium citrate in sterile centrifuge tubes. The tubes were spun at 3,000 revs. for 30 minutes and the plasma removed. The infected cells were then incubated at 37° C. for 14 days and then added as before to 15 ml. of streptokinase broth. Subcultures were plated daily. Results are shown in Table III.

TABLE III

ISOLATION OF *S. TYPHI* FROM INFECTED CELL SUSPENSIONS

No. of Cultures	No. of Positive Isolations
16	12 (after 1 day) 16 (after 2 days)

All 16 gave a positive isolation after two days' incubation. In this case all plasma had been removed so that the effect of antibody and complement could be ruled out, though any phagocytic action might still be expected to play a part.

Incubation of S. typhi in Human Fibrin Clot

Four-ml. volumes of a 1% solution of human fibrinogen were added to 1-ml. volumes of *S. typhi* saline suspension containing approximately 60 viable organisms in sterile 5 in. by ¼ in. tubes. A drop of commercial thrombin was added to each, and after clotting the tubes were incubated at 37° C. for 14 days before being added to streptokinase broth as described. Results of subculture are detailed in Table IV.

TABLE IV

ISOLATION OF *S. TYPHI* FROM INFECTED HUMAN FIBRIN

No. of Cultures	No. of Positive Isolations
30	25 (after 1 day) 30 (after 2 days)

Again positive isolations were obtained from 100% of the infected clots in spite of the preliminary period of incubation at 37° C.

Discussion

Felix (1924), Soman (1932), and Compton and Said (1933) all stress the need for removal of the separated serum before culturing the residual clot. With regard to the isolation of *S. typhi* from blood clot, Felix states: "The culture of *B. typhosus* or *B. paratyphosus A* and *B* from coagulum bile was hardly ever unsuccessful when it was successful from blood bile. The contrary, however, was often the case." He considers that this is due to the removal of bacteriolytic factors when the serum is discarded. We have already shown that the results of clot culture using streptokinase broth are considerably better than those of the routine culture of whole blood using 35 ml. of bile broth for the latter, and that this is due to the presence of bactericidal serum factors in sufficient concentration to produce a bacteriolytic action (Watson, 1954).

No mention appears to have been made, however, of the effect that serum adsorbed on the clot meshwork may have on organisms contained in the clot. The observations presented above

would indicate that these may be of considerable importance. Particularly will this apply where there is little or no lysis of the clot taking place, as in bile broth alone, since there will be no opportunity for dilution of the serum by the volume of medium. It also appears that the bactericidal action of such serum is not limited to the period when serum is present in the clot before retraction occurs, since infected clots added to streptokinase broth immediately retraction was complete all gave positive results. A certain amount of killing is to be expected then, but does not appear to be sufficient to cause complete sterility in the case of an inoculum of 40 to 60 organisms.

The bactericidal action observed after seven days' storage at 37° C. was less than that after 14 days. Mackie and Finkelstein (1932) have shown that the bactericidal action of normal serum against Gram-negative organisms is dependent on the presence of antibody and complement. However, the greater number of negative results after 14 days than after seven suggests that other factors may be responsible since complement tends to disappear in about four days at 37° C. Wright (1942) has been able to demonstrate an increase in the bactericidal power of blood following the addition of organisms to it *in vitro*, and attributes this to the release of factors from the leucocytes. Such factors, and the naturally occurring bactericidins not dependent on the action of complement, may be responsible for the killing observed between seven and 14 days at 37° C.

Whether or not a bactericidal effect will occur in the case of organisms trapped in blood clot will depend on a number of factors, including (*a*) the volume of adsorbed serum, which in turn is dependent on the degree of clot retraction, (*b*) the strength of any bactericidal factors present, and (*c*) the particular strain of *S. typhi* and the number of organisms.

It would seem essential in clot culture techniques in typhoid fever to bring about rapid lysis of the clot in addition to removing as much of the expressed serum as possible.

Summary

Incubation of blood clot infected with *S. typhi* may result in killing the organisms if quick lysis does not occur. Killing was observed in clots stored at 37° C. but not at 4° C. Evidence is presented that serum factors may be adsorbed on the clot meshwork in sufficient amounts to produce a bactericidal effect.

I am indebted to Dr. J. C. Thomas, Provincial Pathologist, Natal Provincial Administration, for his continued interest.

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EFFECT OF CHLORAMPHENICOL ON THE ISOLATION OF *S. TYPHI* FROM THE BLOOD STREAM

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The usually excellent clinical response to chloramphenicol in typhoid fever has been accompanied in practically all the recorded series by a rapid disappearance of organisms from the blood stream. Cook and Marmion (1949) state: "The action of 'chloromycetin' appears to be to arrest the disease at whatever stage it has reached and to sterilize the blood at the same time." Marmion (1952) in a series of 330 cases in Service personnel found that "blood culture almost invariably became sterile within a few hours of starting chloramphenicol therapy." Woodward, Smadel, Ley, Green, and Mankikar (1948) found two cases where it was possible to isolate *S. typhi* from the blood stream after the patients had been on chloramphenicol for 24 hours, but considered this to be unusual.

Our experience has been at variance with that of others, and, following the isolation of *S. typhi* from a case on treatment with the antibiotic, using the clot culture method we have already described (Thomas, Watson, and Hewstone, 1954), we were able to isolate the organism on 16 occasions from 15 cases in a series of 93 cases. In each of these the patients were receiving what were considered to be theoretically adequate doses of chloramphenicol.

Method of Clot Culture

Five millilitres of blood was removed from the antecubital fossa, or from the femoral vein in the case of infants, and allowed to clot. After removal of the separated serum the clot was added to 15 ml. of bile salt broth containing 100 units/ml. of streptokinase, which produces rapid clot lysis. Subcultures were plated daily on to Wilson and Blair's medium. Results are detailed in Table I.

In each of the above cases the chloramphenicol content of the separated serum was estimated using a broth dilution technique against a standard solution of pure chloramphenicol, with *Shigella sonnei* as the test organism. In all 16 instances the serum level lay between 20 and 45 μg./ml. Each of the 16 strains isolated was found to be sensitive to concentrations of chloramphenicol of from 0.5 to 3 μg./ml.

TABLE I
ISOLATION OF *S. TYPHI* FROM BLOOD CLOT

No.	Case No.	No. of Days' Treatment Before Specimen Taken	Last Dose Before Specimen Taken (mg.)	Total Dosage (g.)	No. of Days' Incubation Required
1	1955	10	250	10	2
2	2490	2	500	4	2
3	3179	1	500	2	2
4	3714	2	250	2	3
5	3712	1	500	2	1
6	4192	1	500	1	2
7	5536	8	250	8	2
8	6387	2	500	2	3
9	6078	2	500	3	3
10	6078	11	500	22	1
11	6760	2	500	2	1
12	6757	2	250	1·5	1
13	6756	2	250	3	2
14	65	2	250	3	2
15	6759	3	250	4	3
16	373	1	250	1·5	1

Routine blood cultures were performed at the same time as the clot cultures by adding 8–10 ml. of blood to 35 ml. of bile broth in screw-capped 4-oz. bottles. Again subcultures were plated daily on to Wilson and Blair's medium. Only one positive isolation was obtained in this way. The reason for this is presumably that the effective bacteriostatic concentration of chloramphenicol was still sufficient in the blood-medium mixture to prevent growth of viable organisms in addition to the bactericidal action of serum factors not sufficiently diluted by the volume of medium used (Watson, 1954). In the case of the clot cultures, however, removal of the serum before culture has eliminated both these factors and the small quantity of each left in the volume of serum retained in the clot meshwork was not sufficient to prevent growth of viable organisms when diluted out in 15 ml. of medium.

Reasons for Positive Isolations from Clots

The action of chloramphenicol is primarily a bacteriostatic one (Smadel, 1950), though Bliss and

Todd (1949) have shown a slow bactericidal action. If the observations of Smadel are correct it seemed possible that the explanation of the positive clot cultures might be the presence of circulating viable organisms which could be demonstrated by removal or suitable dilution of the antibiotic. Another explanation presented itself, however, namely, that viable organisms might be phagocytosed *in vivo* and be protected from the action of chloramphenicol by virtue of their intracellular position. Support for this observation is to be found in the work of Rous and Jones (1916), who demonstrated protection of intracellular forms of *S. typhi* from the action of specific antiserum and potassium cyanide. Adams (1939) has demonstrated intracellular forms of *S. typhi* in plasma cells in intestinal and mesenteric lesions. Magoffin and Spink (1951) have shown protection of intracellular forms of *Br. abortus*, phagocytosed by cells of the buffy coat, from the bactericidal action of streptomycin.

Demonstration of Protection of Intracellular Forms

This problem was investigated using streptomycin in a manner somewhat similar to that described by Magoffin and Spink (1951). Streptomycin was chosen, since the authors have shown that with chloramphenicol and the other broad spectrum antibiotics death of extracellular forms occurs too slowly to allow protection of intracellular forms to be demonstrated before death and disintegration of the harbouring leucocytes.

Method.—Twenty millilitres of blood from a donor was added to 2 ml. of 3.8% sterile sodium citrate. After centrifuging at 3,000 revs. for 20 minutes the buffy coat was transferred to a sterile Wintrobe tube and spun again. The buffy coat was then removed and divided into two equal parts. One-half ml. of a 1 in 1,000 saline dilution of an overnight broth culture of a freshly isolated strain of *S. typhi* was added to one part in a sterile 3 in. by ½ in. tube, which was then incubated in a water bath at 37° C. for four hours. Gram-stained films then showed the presence of intracellular forms. Five millilitres of streptomycin solution containing 100 μg./ml. was then added. A control tube containing the other part of the buffy coat, *S. typhi* suspension, and streptomycin was set up but without preliminary incubation of the buffy coat and bacterial suspension.

Both tubes were incubated at 37° C. in a water bath. At varying periods of incubation 0.5 ml. amounts were removed and serial tenfold dilutions prepared in saline. Plate count estimations of surviving organisms were then made. Results are detailed in Table II.

TABLE II

ACTION OF STREPTOMYCIN ON INTRACELLULAR AND EXTRACELLULAR FORMS OF *S. TYPHI*

Time of Incubation (Hours)	Dilution	Colony Counts per 1 ml. Vol.	
		Test Sample	Control Sample
4	1/10	310	30
	1/100	63	4
	1/1,000	14	Nil
8	1/10	230	"
	1/100	42	"
	1/1,000	8	"
12	1/10	240	"
	1/100	31	"
	1/1,000	6	"
24	1/10	190	"
	1/100	30	"
	1/1,000	4	"

Discussion

The results in Table II demonstrate clearly that where intracellular forms were present growth was significantly better than in the control series where no phagocytosis took place. These findings, in conjunction with the observations of Adams (1939), appear to supply a rational explanation of the results in Table I. It is probable that where phagocytosis occurs *in vivo* a proportion of the phagocytosed organisms remain viable for a certain length of time and are protected from circulating antibiotic. In this connexion it is interesting to note that no demonstration of intracellular penetration by chloramphenicol is as yet forthcoming. The work of Smith, Worrel, and Swanson (1949) suggests that a barrier exists at the cell wall. Glazko, Wolf, and Dill (1949), concerning the distribution of chloramphenicol in red cells, state: "The binding is probably due to adsorption rather than slow diffusion through the cell membrane." This has been our experience also.

Rous and Jones (1916) showed that intracellular protection was dependent on the cells being alive. When death occurred changes in the permeability of the cell walls led to the contained organisms being exposed to the action of bactericidal factors.

Though the isolation of *S. typhi* from the blood stream of cases on therapeutically adequate doses of chloramphenicol has not been reported to any extent by others, *S. typhi* has been isolated after death from various tissues (Reilly, Compagnon, Tournier, Bastin, and du Buit, 1950; Marmion, 1952). If the observations of Adams (1939) are correct, it is probable that intracellular forms of *S. typhi* were present in these tissues. It may be that such foci of organisms are responsible for maintaining an intermittent bacteraemia and that, in view of the primarily bacteriostatic action of chloramphenicol, positive isolations will be obtained during this time provided there is adequate dilution or removal of the antibiotic.

Only one positive blood culture was obtained as against 16 positive clot cultures. This suggests that the volume of culture medium in the former, 35 ml., was too small, since with adequate dilution of chloramphenicol and serum factors an equal number of positive blood cultures would be expected. We have already shown that the results of clot culture using streptokinase bile broth are considerably superior to the routine blood culture results using this volume of medium, and that this is due to a sufficient concentration of bactericidal serum factors in the latter (Watson, 1954).

If the intermittent release of intracellular forms in tissue into the blood stream is the true explanation of the results in Table I, it is necessary to postulate some mechanism of release from the cells. This may follow death of the cells, but Wilson (1953) has recently shown by phase-contrast microscopy ingestion followed by egestion of viable forms of streptococci. It may be that a similar mechanism operates in this instance. The results in Table II are of course not directly applicable to the problem of the effect of chloramphenicol *in vivo*, but they do provide a possible explanation for the positive cultures reported in Table I.

Summary

Isolation of *S. typhi* from blood clot in 16 instances from 15 cases receiving theoretically adequate doses of chloramphenicol is presented.

Intracellular protection of phagocytosed forms of *S. typhi* from the action of antibiotics (streptomycin) is discussed.

A possible explanation of the positive clot cultures is given.

I am indebted to Dr. J. C. Thomas, Provincial Pathologist, Natal Provincial Administration, for his interest and encouragement. I wish to thank Messrs. Parke, Davis & Co. for a supply of pure crystalline chloramphenicol.

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OPTOCHIN IN THE IDENTIFICATION OF *STR. PNEUMONIAE*

BY

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In 1911 Morgenroth and Levy showed that mice could be protected against pneumococcal infection with "optochin" (ethylhydrocupreine hydrochloride). The clinical use of this agent was soon abandoned as it was found to be toxic and, in doses which could be tolerated, did not influence the course of lobar pneumonia (Moore and Chesney, 1917; White, 1938). Subsequent *in vitro* studies showed that of the streptococci, *Str. pneumoniae* was the most sensitive (Moore, 1915), and that as low a concentration as 1 in 50,000 incorporated in blood agar would inhibit growth (Mørch, 1943).

Bile solubility has been generally regarded as the most reliable test for the differentiation of pneumococci from other haemolytic streptococci (Wilson and Miles, 1946). The test, however, is time-consuming and sometimes gives equivocal results.

This paper describes an investigation to determine whether "optochin" sensitivity can be used for the identification of *Str. pneumoniae* in place of the bile solubility test. A large number of alpha haemolytic streptococci were examined to ascertain the correlation between these tests. Mouse virulence was used as the final criterion of the clinical significance of some of the strains.

Materials and Methods

Origin of Strains.—Cultures from single colonies of alpha haemolytic Gram-positive cocci were obtained from blood agar cultures of sputa, throat swabs, and pus submitted to this department for routine investigation. A number of strains of *Str. pneumoniae* were received from other laboratories.

Bile Solubility Test.—About 1 ml. of a saline suspension of growth from half a blood agar plate was distributed in two Dreyer tubes to one of which was added 2 drops of a 10% solution of sodium taurocholate of proved activity and to the other 2 drops of saline. The test was regarded as positive if there was clearing in the taurocholate tube after incubation in the 37° C. water bath for one and a half hours. Suspensions were consistently of *p*H 7.2.

Tests which gave incomplete clearing in the taurocholate tube were repeated by both authors and checked by an independent observer.

Optochin Sensitivity

The Ditch Method.—This method was given in a personal communication from Dr. Landsman.

A ditch about 1 cm. wide was cut across the centre of a blood agar plate and filled with 1 in 50,000 "optochin" in nutrient agar. The test organisms and a known sensitive strain were streaked up to the ditch.

The Disc Method.—Discs of filter paper 8 mm. in diameter were sterilized by dry heat at 160° C. for one and a half hours. Each disc received 0.02 ml. of a 1 in 4,000 solution of "optochin," and was then dried at 37° C. This concentration of "optochin" gave inhibition zones with *Str. pneumoniae* slightly smaller than the ditch method. The organisms for test and a known sensitive strain were streaked radially on a blood agar plate, a disc was placed in the centre, and readings were made after overnight incubation at 37° C.

Similar results were obtained with strips of filter paper 8 × 1 cm. which had been dipped into the solution, drained of excess fluid, and dried as above. Defibrinated horse blood agar containing 0.005% para-amino-benzoic acid was used for all experiments. Lysed blood agar without this addition and oxalated blood agar were shown to give similar results.

Ethylhydrocupreine hydrochloride ("optochin," Howards, Ltd., Ilford) is freely soluble in water. The base is insoluble. Solutions of 1 in 500 and 1 in 4,000 have proved stable in the refrigerator and at room temperature. The 1 in 4,000 solution was unaffected by autoclaving at 15 lb. for 30 minutes. Sterilization of the discs at 160° C. cannot be combined with drying of the solution on them, as a considerable loss of potency results. Discs made from 1 in 4,000 solution dried at 37° C. and stored in screw-capped bottles were stable for nine months in the refrigerator and at room temperature in daylight.

Mouse Virulence Test.—Mice were inoculated intraperitoneally with 0.5 ml. of an overnight serum broth culture. Deaths within seven days were recorded.

From dead animals, cultures of the heart blood were made, and stained smears of heart blood and peritoneal exudate examined for the presence of *Str. pneumoniae.*

Experimental

Complete insensitivity to "optochin" was shown by growth of organisms right up to the edge of the disc or ditch. Sensitivity was of two grades, which could be very easily distinguished. Full sensitivity resulted in a zone of inhibition of more than 5 mm. from the edge of the disc. Partially sensitive strains gave very small zones not exceeding 2 mm.

TABLE I

Strain	No. of Strains	Inhibition from Edge of Optochin Disc in mm.		
		Nil	1 or 2	5 to 9
Bile soluble ..	243	None	1	242
„ insoluble ..	452	434	17	1

Table I records the "optochin" sensitivities of 243 bile-soluble strains and 452 bile-insoluble strains. Of the former, all except one were fully sensitive to "optochin"; the exception was partially sensitive and was found to be completely avirulent for the mouse. Of the bile-insoluble strains, only one was fully "optochin" sensitive, but 17 were partially so. The wholly sensitive strain and 13 of the 17 partially sensitive strains were tested for mouse virulence and all were found to be completely avirulent.

There is thus almost complete correlation between bile solubility and full "optochin" sensitivity, but 2.4% of the 695 strains examined showed slight "optochin" sensitivity in spite of being bile insoluble. As all of these which were tested were found to be completely avirulent for mice it is reasonable to assume, either that they were not true pneumococci, or that they represented completely rough variants unlikely to be of any clinical significance.

The proportion of bile-insoluble streptococci giving visible evidence of slight sensitivity to "optochin" can be reduced by reducing the concentration of "optochin" used. This, however, is accompanied by a reduction in the size of the zone of inhibition given by true pneumococci. The relation between "optochin" concentration and zone size was determined by tests on 55 strains of *Str. pneumoniae* by the disc method, with various concentrations of "optochin." The results are tabulated in Table II.

As inhibition zones of 2 mm. might be missed by inexperienced observers it would be unwise to use an "optochin" concentration of less than 1 in 8,000 for routine diagnostic work. In practice, we have found that a concentration of 1 in 4,000 is satisfactory and does not give rise to any difficulty in distinguishing between the inhibition zones of pneumococci and of the anomalous strains of bile-insoluble streptococci. As an additional check we also record the size of the inhibition zone of a control *Str. pneumoniae* for each disc. The sensitivity of this control strain has not altered with daily subculture on blood agar for one year.

TABLE II

Optochin	Limits of Zones of Inhibition of *Str. pneumoniae* (mm.)
1/4,000	5 to 9
1/8,000	4 „ 8
1/16,000	2 „ 6

Experimental Dissociation of Bile Solubility and Optochin Sensitivity

In view of the natural occurrence of strains showing no correlation between "optochin" sensitivity and bile solubility an attempt was made to separate these characteristics experimentally.

Str. pneumoniae type I (N.C.T.C. 7465) was shown to be bile soluble, "optochin" sensitive, and mouse virulent. A series of dilutions of "optochin" in 5% serum broth was seeded from an overnight serum broth culture of this organism. After 24 hours' incubation the culture containing the highest concentration of "optochin" and showing turbidity was used to inoculate a second series of "optochin" serum broths. This procedure was repeated through a number of serial subcultures.

After two subcultures the organism became "optochin" resistant. In the standard disc test it grew up to the disc, and its sensitivity, tested by titration in serum broth, had decreased from 1 in 500,000 to 1 in 50,000. It was bile soluble.

After eight subcultures "optochin" sensitivity had fallen to 1 in 5,000, though the organism still remained bile soluble. At this stage the mouse virulence test was repeated. To exclude the possibility of a bacteriostatic effect from the presence of traces of "optochin," the pneumococci were washed in peptone water and resuspended before injection. Furthermore the test was repeated after three serial subcultures in serum broth to eliminate "optochin" which may have been adsorbed on the cells. In each test the mice survived, indicating an association between loss of "optochin" sensitivity and loss of mouse virulence, with dissociation from the bile solubility.

A second attempt to separate the properties of bile solubility and "optochin" sensitivity of the original strain (N.C.T.C. 7465) was made by serial culture in 10% type specific antiserum in serum broth. Through 14 daily subcultures the organism remained bile soluble and "optochin" sensitive, though losing mouse virulence.

Application

The colonial morphology of some strains of *Str. pneumoniae* and *Str. viridans* is very similar. When two such strains appear in a mixed culture either may go unrecognized and the proportion of each may be difficult to assess. In a routine diagnostic laboratory it is not always practicable to avoid this error by testing numerous colonies from each plate. An alternative simple method is to place an "optochin" disc on the pool of inoculum of the original blood agar plate. The presence of large numbers of pneumococci will be revealed by a zone of sparser growth round the disc due to inhibition of the pneumococci. The effect is striking even when fairly heavy growth of other organisms, including *Str. viridans*, occurs right up to the edge of the disc. Plates incubated anaerobically show the effect even more clearly, because many bacterial species are inhibited under these conditions. Nor does the addition of 10% carbon dioxide to the atmosphere affect the phenomenon. A confirmatory Gram-stained film should be examined before making a report. Alternatively several single colonies may be tested by the "optochin" disc or strip on one blood agar plate.

Summary

"Optochin" sensitivity offers a satisfactory means of identification of *Str. pneumoniae*. It is less time-consuming than the bile solubility test.

Of 695 strains of alpha haemolytic streptococci tested, only two showed lack of correlation between bile solubility and full "optochin" sensitivity.

The "optochin" disc method facilitates detection of *Str. pneumoniae* on overnight plate cultures of sputum or pus containing a mixed bacterial flora.

One strain of pneumococcus was experimentally rendered "optochin" insensitive while retaining its bile solubility.

We wish to thank Professor Wilson Smith, F.R.S., and Dr. E. J. Stokes for their guidance and encouragement in the preparation of this paper. We are grateful to those who have sent us strains of *Str. pneumoniae*, especially Mr. E. Y. Bridson.

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THE ESTIMATION OF EVANS BLUE IN PLASMA

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Direct colorimetric estimation of Evans blue in plasma is invalidated by the presence of opalescence or haemolysis. Various methods have been designed to overcome this difficulty and in general they have only been partially successful. The butanol method of Harington, Pochin, and Squire (1940) is time-consuming, yields variable dye recoveries, and is capable of dealing with traces of haemolysis only. The Crooke and Morris (1942) technique, involving protein precipitation with a mixture containing ethanolic phosphotungstic acid, is not readily applicable to haemolysed samples and the precipitant induces some instability in the dye. Recovery of the dye also tends to be inconsistent in the chromatographic method of Morris (1944) which uses aluminium hydroxide as absorbent. More recently a notable advance in chromatographic technique has been achieved by Allen (1951), who employs shredded tissue paper cellulose for sorption of the dye, and obtains consistent recoveries closely approximating to 97%.

Trials of various forms of cellulose available to us at the time of beginning work on plasma volume estimations showed that, while some samples were capable of highly efficient separation of Evans blue from plasma, the subsequent elution invariably presented some difficulty. However, an easily prepared form of degraded amorphous cellulose was found readily amenable to both sorption and elution processes, and it was further established that the whole of the procedure was facilitated by the use of elevated temperatures. These findings became the basis of the method to be described.

Method

Reagents.—The following are required :—

Degraded Amorphous Cellulose.—A quantity, 3 g., of acetone soluble cellulose acetate (Hercules Powder Co. Ltd., Grade PM3E) is placed in a 500-ml. conical flask and dissolved with the aid of continuous shaking in 100 ml. of 95% acetone. While the contents of the flask are gently swirled N NaOH is added slowly from a burette until the reaction is just alkaline. The precipitated cellulose is centrifuged at 1,200 r.p.m. for three minutes. After decanting the supernatant liquid the cellulose is washed three times with 100 ml. of physiological saline, each time lightly stirred to an even suspension with a glass rod, and then separated by centrifuging. The final suspension is made up in 250 ml. of physiological saline and stored in a refrigerator. Preparations not more than one week old should be used to ensure consistent dye recovery.

Celite (Johns-Manville, No. 545).—The absorption column is prepared from 7 ml. of cellulose suspension with about 50 mg. of celite added just before use.

Chemical Cotton (Hercules Powder Co. Ltd., Grade 13).—This most severely degraded form of cotton is used as a filter plug supporting the column.

Teepol (Shell Chemicals Ltd., Grade 530).—(*a*) Dilute "teepol," equal parts by volume of "teepol" and distilled water ; (*b*) "teepol"-saline, 1 in 200 dilution of "teepol" in physiological saline.

Acetone-water.—35% v/v.

Evans Blue (G. T. Gurr Ltd.).—Evans blue, 100 mg., is dissolved in 100 ml. of pyrogen-free water, autoclaved, and stored in 25-ml. sealed ampoules.

Apparatus.—The apparatus consists of a filter and a funnel heater jacket.

The Filter.—This is made by fusing two lengths of glass tubing with bores of 2 cm. and 5 mm. respectively. The wider tube is tapered to form the neck of the filter and junction with the narrower tube, whose lower end is cut off obliquely. The wide upper part of the filter should measure 10 cm. in length and the lower narrow end about 4 cm.

Funnel Heater Jacket.—A standard copper jacket is used, 6 in. internal diameter across the top.

Technique.—This is as follows :—

Injection of Dye and Blood Sampling.—Twenty millilitres of the dye solution is injected intravenously into one arm from a syringe, the delivery of which was checked by weighing. Exactly 10 minutes later a 10-ml. blood sample is taken from the opposite arm without venous stasis, and prevented from clotting by use of Wintrobe's anticoagulant mixture. The packed cell volume is determined by the standard Wintrobe method and the remainder of the dyed sample is centrifuged for 15 minutes.

Determination of Evans Blue.—A plug of chemical cotton is packed into the neck of the filter sufficiently tightly to allow a flow rate of approximately 1 ml. per minute when using a 10 cm. head of distilled water. The filter is then suspended by a clamp in the centre of the funnel heater jacket in which the water is kept very gently boiling. The position of the filter is adjusted so as to maintain its contents at approximately 45° C. throughout the sorption and elution stages of the procedure. The suspension used to form the column and all washing and eluting fluids are first heated to 50° C. in a water bath immediately before their use on the column.

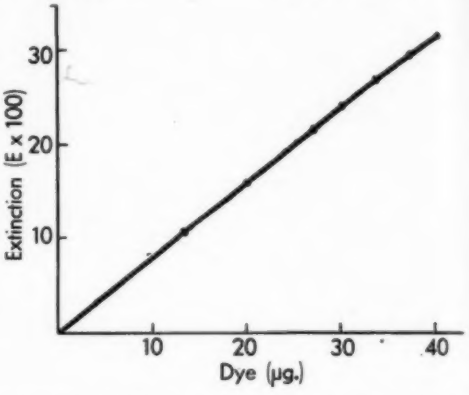
Celite suspended in "teepol"-saline is transferred to the filter, using a wide-bore Wright's pipette, and allowed to settle under gravity to form a layer 1.5 cm. deep. When about 1 cm. of fluid remains above the celite, the cellulose–celite suspension is added and washed twice with 10 ml. of "teepol"-saline. This forms an adsorbing layer about 7 mm. deep on a lower layer of pure celite. At no time is the column allowed to be free from fluid, nor, once the column is formed, should the fluid depth above the surface ever exceed 2 cm. Subsequent additions of fluid to the column should all be run carefully down the sides of the funnel.

To 4 ml. of dye-plasma contained in a 25-ml. beaker is added 2 ml. of dilute "teepol," mixing being effected by gentle rotation so as to avoid bubble formation. The mixture is warmed to 50° C. for 10 minutes and transferred to the column as completely as possible with the aid of a Wright's pipette. When the plasma mixture passes through the column so that only a 3–4 mm. layer remains above the surface, 2 ml. of pre-heated "teepol"-saline is used to rinse the beaker and is then transferred to the column, the process being repeated with a further 2 ml. of "teepol"-saline, and then finally with 1 ml. of this washing liquid. Just before the last rinse recedes into the column the filter tip is rapidly washed with a jet of distilled water to remove any trace of protein, and the elution acetone is added so as to maintain a layer of about 10 mm. above the cellulose. As the acetone penetrates, the dye moves as a sharp blue band down the column. When it enters the lower celite layer, the eluate is collected in a graduated 10-ml. cylinder until a volume of 6.5 ml. is reached. This is cooled to room temperature and made up to a volume of 7 ml. with 35% acetone. After thorough mixing the intensity of colour is read immediately in a Spekker absorptiometer against a blank of aqueous acetone, using Ilford filters 607. The total time taken, from obtaining the dyed sample of blood to the final reading, is approximately one hour.

Results

Light Absorption of Evans Blue in 35% Acetone.—The calibration curve (Fig. 1) was made from serial dilutions, prepared by adding from an Agla micrometer syringe known amounts of a standard solution of Evans blue, to make a total volume of 7 ml.

FIG. 1.—Relationship between optical density and amount of dye in 7 ml. of 35% aqueous acetone (Spekker absorptiometer: Ilford 607 filter).



These results were not influenced by the incorporation of up to 2 ml. of washing liquid in the 7 ml. volume. Thus in estimations on plasma the small volume of washing liquid passing into the receiver does not affect the readings.

Recovery of Evans Blue from Non-haemolysed Plasma.—Thirty-two estimations were carried out on three different concentrations of dye in samples of fresh plasma of widely different optical character, ranging from very clear to grossly turbid. The results (Table I) show that there is no appreciable difference in the mean recoveries and standard deviations. These concentrations cover the range most commonly encountered when estimating plasma volumes in man, using 20 mg. doses of Evans blue intravenously.

TABLE I
RECOVERY OF DYE FROM NON-HAEMOLYSED PLASMA

No. of Determinations	Evans Blue Present in 4 ml. Plasma (µg.)	Recovery (%)		Standard Deviation
		Mean	Range	
6	30·8	97·3	97·0–97·5	0·33
20	26·7	97·0	96·2–97·7	0·26
6	23·5	97·3	96·7–97·8	0·48

Recovery of Evans Blue from Haemolysed Plasma.—To three test-tubes each containing 4 ml. of fresh plasma were added haemolysed red cell solutions to give respectively a content of 1 mg., 2 mg., and 4 mg. of haemoglobin before the addition of Evans blue. As is shown in Table II, these quantities of haemoglobin did not alter recovery of the dye. The blood pigment was easily

TABLE II
RECOVERY OF DYE FROM HAEMOLYSED PLASMA

Hb Present (mg.)	Evans Blue in 4 ml. Plasma (μg.)	Recovery (%)
0	27·7	97·2
1·0	27·7	96·7
2·0	27·7	96·7
4·0	27·7	97·2

visible as it passed through the column with the plasma during the adsorption and washing stages.

Results on Normal Males.—Plasma volume estimations were carried out on 10 healthy male subjects of 21 to 28 years of age. Three estimations were made on each subject at intervals of approximately one week. Conditions for each estimation were standardized by admission to hospital for one night, ensuring 10 hours' rest in bed.

Nothing was allowed by mouth after 8 p.m. apart from water and fruit drinks and the test was performed at the same hour each morning before rising. The plasma volume was determined by the present method and also by direct colorimetry as described by Mollison (1951). The latter method necessitated taking a sample of blood before the injection of the dye; this was also required on the second and third estimations by the chromatographic method as the concentration of the residual dye* in these pre-injection samples had to be determined and taken into account in the final estimation. The results of this series of cases without obvious cloud or haemolysis is shown in Table III, those for the chromatographic method being corrected for a 3% loss in the process. The mean by the chromatographic method described was 44.9 ml./kg., and by direct colorimetry was 47.1 ml./kg.

Only one case, whose plasma was very cloudy, was examined by the direct method to see how it compared with the elution method. Two determinations were made at an interval of three weeks giving values of 65.8 and 69.1 ml./kg. as against 47.1 and 47.3 ml./kg. for the elution method, thus showing a much wider discrepancy of results than any obtained on clear samples.

Discussion

The method described represents a convenient procedure for isolating the dye from plasma and measuring its concentration in simple solution in such a way as to obviate completely the vitiating effects of haemolysis and lipaemia upon the estimations. It compares favourably with that of Allen to which reference has already been made. Our experience with various preparations of shredded tissue paper, and also of cellulose powder specially prepared for chromatography, has shown the degraded form of amorphous cellulose regenerated from cellulose acetate to be more suitable both in respect of absorption of dye and its subsequent elution. Whereas 130 mg. of tissue paper cellulose is required in the Allen technique, about 50 mg. of amorphous cellulose suffices for the present type of absorption column. The less cellulose required to constitute the column the greater the ease of complete elution. Slight losses have been encountered in washing the column with saline alone. These were avoided by the "teepol"-saline washing used in the present method, but even in these conditions it is essential to keep the total amount of washings to a minimum. At ordinary room temperatures the elution process has occasionally left a visible trace of blue on the surface of the column. The heating of the atmosphere surrounding the column has completely eliminated this factor as a source of slightly

TABLE III
COMPARISON OF PRESENT ELUTION AND DIRECT METHODS OF ESTIMATING PLASMA VOLUME

Subject	Weight (kg.)	Plasma Volume (ml./kg.)		P.C.V. (% Uncorrected)
		Eluted	Direct	
1	66·4	60·5	58·0	45
		56·6	57·6	45
		57·9	62·4	45
2	66·8	43·9	40·9	47
		41·0	44·3	47
		41·9	46·4	47
3	73·6	50·2	53·1	46
		46·9	48·9	46
		50·5	52·1	45
4	64·1	42·9	44·6	47
		42·7	43·7	47
		43·9	47·5	47
5	63·2	43·6	45·7	48
		48·9	52·1	46
		48·0	53·8	48
6	69·1	42·7	45·9	46
		41·1	47·4	47
		45·2	46·2	45
7	67·3	47·9	49·4	43
		51·0	46·4	44
		52·8	53·3	42
8	68·2	38·8	41·9	50
		36·4	40·3	51
		39·0	40·7	50
9	60·9	39·0	40·0	54
		36·5	39·6	52
		37·6	37·7	53
10	76·4	36·9	40·7	47
		40·6	43·5	48
		42·2	48·0	47
		Mean 44·9	Mean 47·1	Mean 47·2

* With the present technique dye has been detected in the plasma as long as 36 days after a single injection.

inconsistent results. The alternative form of heating by surrounding the column with warm water has been found to have the disadvantage in the elution process of tending to create some channelling in the area of contact with the glass funnel. Most of the preliminary work was done in sintered glass filter tubes. Under these circumstances a few tiny particles of celite and cellulose were carried through with the eluate, a result attributable to the use of the detergent. The substitution for a sintered glass disc of a cotton plug made of severely degraded material provided clearer eluates for a given filtration rate. To obtain complete clarity with the use of sintered glass funnels the disc had to be of such fine porosity as to slow down the whole process to an inconvenient degree. Further, prolonged contact of the dye with the constituents of the column generally led to slight loss in the recovery of dye so that it was essential to resort to a process in which the elution rate was fairly rapid.

Because coloured impurities in Evans blue introduce an error into plasma volume determinations (Leeson and Reeve, 1949), a careful preliminary search was instituted for a preparation which contained only traces of the red impurity, a common contaminant of commercial products. The results recorded here are those which have been obtained by using a dye containing little more than a trace of this red component. It was of such small concentration that its presence did not affect the maximum absorption of light by more than 0.5%.

The efficiency of the combined sorption and elution process may be gauged by the fact that it was found possible to detect concentrations of dye as low as 10 μg. per 100 ml. plasma as an easily visible faint blue on the surface of the column, and that at the end of every elution process the column when compared with a freshly prepared unused column showed no detectable difference in colour.

The results of the estimations performed on normal male subjects show that the mean of the chromatographic method was 2.2 ml./kg. less than the mean obtained by direct colorimetry. Moreover the range of values on each individual subject was generally appreciably less by the present method than by the direct method. The discrepancies between the two sets of results may be partly explained by the fact that a single standard calibration curve was used for all the direct determinations, being prepared from known quantities of dye added to pooled fresh clear plasma. Had a standard been prepared in the corresponding pre-injection sample at each estimation the results might have been closer, but this would have involved taking unduly large blood samples.

Our mean value of 44.9 ml./kg. by the chromatographic method is in close agreement with the mean value of 44.7 and 45 for normal males reported by Noble and Gregersen (1946) and Henschel, Mickelsen, Taylor, and Keys (1947) respectively. The mean haematocrit values of these workers were also nearly identical with our own.

Summary

A chromatographic method is described for estimating Evans blue in plasma using a column of degraded amorphous cellulose. The dye separates as a narrow band on the surface of the column and is subsequently eluted with aqueous acetone. Consistent recoveries of 97% are obtained and the analyses are not affected by the presence of opalescence or haemolysis. Comparative results of estimating plasma volume by this elution method and the direct method on normal male subjects are recorded.

We have to express our warmest thanks to Messrs. Hercules Powder Co. Ltd. for placing at our disposal specimens of high-grade cellulose acetate and chemical cotton, and to Messrs. Shell Chemicals Ltd. for a range of samples of anionic detergents.

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THE EFFECT OF DIET UPON THE RESPONSE TO ORAL ANTICOAGULANTS

BY

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It is not uncommon in clinical practice to meet with patients who do not respond in the expected manner to the usual therapeutic doses of the oral anticoagulants. Some appear to be unusually sensitive, while others, who are encountered more frequently than these hyper-reactors, prove abnormally resistant to the action of these drugs. While carrying out clinical trials with "tromexan" (Burt, Payling Wright, and Kubik, 1949) and with "dindevan" (Payling Wright, 1954, in press) in groups of hospital patients with various thrombotic disorders, it was found that the proportion who manifested resistance was about one in six of those treated. The care of such patients furnishes some of the chief anxieties to the physician using anticoagulant drugs, for they may present difficulties of two distinct kinds. In the first, when the dose is increased in the effort to overcome the resistance, the drug may exhibit a cumulative effect, and occasionally a sudden, rapid, and full response occurs on the third or fourth day which depresses the prothrombin activity to such a degree that severe, spontaneous haemorrhage supervenes. In the second, at no stage is any satisfactory reduction of the prothrombin level achieved, so that much valuable time may be lost. In this group of patients the thrombus may enlarge during the period of ineffective treatment—a deterioration which naturally has a detrimental effect upon prognosis.

The causes of these variations in response to oral anticoagulants are not understood at the present time, and few observations of an experimental kind have been made which throw light on the nature of the response defect. Three questions may be posed: Is resistance due to failure of absorption from the gut, so that the drug never enters the blood stream? Is it due to failure of the coagulation mechanism within the body to be affected by the circulating anticoagulant? Or is it dependent upon fluctuations in concentrations of vitamin K, which can modify the degree of hypoprothrombinaemia produced by a specific amount of anticoagulant? Observations by Waldron, Beidelman, and Duncan (1951), Fullerton, Davie, and Anastasopoulos (1953), and recently by Duguid (1954) on the relationship of high fat in the diet to changes in the coagulation mechanism and the onset of intravascular thrombosis suggest that the diet, and especially the lipids, might, under certain circumstances, influence the response to oral anticoagulants. The following series of experiments was therefore undertaken in an attempt to elucidate the dietetic aspect of the problem and to try to answer the questions as to the nature of the failure of response in patients resistant to oral anticoagulant therapy.

General Methods and Materials

Young male albino rats, weighing between 150 and 250 g., were used throughout the following experiments. Each group comprised not less than 50 animals, 25 receiving one of two special diets (vide infra). All other substances and drugs were administered by stomach tube while the animals were under light ether anaesthesia. Prothrombin estimations were carried out by the Quick single-stage technique, with thrombokinase (Geigy) as the thromboplastic source. Blood for these determinations was obtained by puncture of the vena cava while the animals were under deep nembutal anaesthesia. The anticoagulant used throughout was bis-3-3′-(4 oxycoumarinyl) ethyl acetate.* This anticoagulant, which is insoluble in neutral solution, was administered as a stable suspension prepared by grinding the active substance with 4% methyl ethyl cellulose in water.† The quantities were adjusted so that 0.75 ml. suspension contained 100 mg. "tromexan."

Two diets, listed in the table on the following page, together with water, were supplied to the animals in unrestricted amounts. During the observation periods no rat lost weight significantly.

* "Tromexan," Pharmaceutical Laboratories, Geigy Ltd.
† "Cellofas A," Imperial Chemical Industries, Ltd.

	High Fat Diet A (g.)	Low Fat Diet B (g.)
Casein	10	10
Yeast	5	5
Salt mixture (Glaxo)	3	3
Starch	51	79
Cod liver oil	1	1
Lard	30	—
Olive oil	—	2

In addition to these diets 0.5 ml. of a supplementary vitamin mixture was given daily by stomach tube to each rat. This mixture contained:

Thiamine HCl	20 μmg.	in 0·5 ml.
Pyridonine	20 ,,	
Riboflavine	25 ,,	
Ca pantothenate	100 ,,	
Aq. kapilon	5 mg.	

The techniques for each group of experiments, together with their results, will, for clarity, be set out separately.

Group 1: Effect of the Anticoagulant on the Blood Picture

Batches of 25 rats of known weights were put upon one of the two diets, A or B, for one week. At the end of this period their red cell counts and haemoglobin percentages were determined on blood drawn by tail clip. Each animal then received 100 mg./kg. body weight "tromexan" daily for five consecutive days by stomach tube. Under full anaesthesia, blood was withdrawn from the vena cava for further erythrocyte counts, haemoglobin, and estimation of mean corpuscular volume. On each day during the administration of anticoagulant, both faeces and urine were tested for the presence of blood; if detected, the animal was discarded. This precaution was taken, since occult blood loss would interfere with the interpretation of results.

Results.—The blood picture findings before and after five days' treatment with "tromexan" are set out below. The figures in brackets give the percentage change from the initial figure which took place during this period.

	Red Cell Count (m.)		Haemoglobin (g.%)		M.C.V. (cu.u)
	Initial	Terminal	Initial	Terminal	Terminal
Diet A	7·15 (100%)	6·47 (90%)	13·9 (100%)	12·0 (86%)	65·6
,, B	6·96 (100%)	6·02 (87%)	13·7 (100%)	10·9 (79%)	66·4

A small but definite fall in the erythrocyte count and haemoglobin percentage followed the administration of the anticoagulant, the effect being somewhat more marked in those animals kept on diet B. This trend was probably due to minor haemorrhages into the tissues, red cells thus being lost to the circulation. Histological sections of liver, kidney, spleen, and bone marrow from animals on both diets demonstrated the presence of such haemorrhages, which were too small to be detected with the naked eye at necropsy. The kidney, and, to a lesser extent, the liver, were the chief sites of these haemorrhagic areas and, as might be inferred from the changes in the peripheral blood, were more frequently found in the rats fed on the low-fat diet. The normal corpuscular volume found after the administration of the anticoagulant offers further confirmation that there is no change in the type of haemopoiesis following its ingestion for a short period.

Group 2: Effect of Diets A and B on Prothrombin Levels

After two weeks on the respective diets the prothrombin times of batches of rats were determined on vena caval blood to ascertain whether the regimen had any significant effect upon the coagulation mechanism. For control, prothrombin times of rats kept on the mixed diet provided in the stock breeding rooms, supplemented with green vegetables and milk, were used.

Results.—The effect upon the prothrombin times of the two diets compared with normal controls was small. The high fat intake tended to depress the coagulation mechanism, an observation similar to those of other workers.

	Controls	Diet A	Diet B
Prothrombin time (sec.)	14·0	13·6	14·2
Range (sec.)	13·0–14·5	12·5–14·0	13·0–15·0

Group 3: Average Lethal Doses of Anticoagulant for Rats on High- and Low-fat Diets

Further batches of rats were kept on the diets for one week, at the end of which time each received 100 mg./kg. body weight "tromexan" daily until death. From the group results, the average lethal dose for animals in each batch was obtained.

Results.—It was found that the average lethal dose for those animals maintained on diet A (high fat) was 1.182 g. "tromexan." That for animals on diet B was almost half this amount, being only 0.595 g. It seemed, therefore, that the high-fat content of the diet had a saving effect for the animals, fatal haemorrhage being more rapidly produced when fat was absent from the diet.

Group 4: Effect of Single Dose of Anticoagulant in Rats on High- and Low-fat Diets

After one week on their respective diets the animals were given a single dose of 150 mg./kg.

body weight "tromexan." Their prothrombin times were determined 24 hours later.

Results.—The effect of the anticoagulant, given on one occasion only, differed considerably in the two series. The average prothrombin time for rats on diet A was 18.54 sec., while that for those on diet B was 22.73 sec. The marked divergence in the responses demonstrates in a striking manner that the ingestion of fat protects the animals from the anticoagulant and reduces their hypoprothrombinaemic response, an observation already noted in a different form in Group 3.

Group 5: Effect of Antibiotic Sterilization of the Gut on Response to Anticoagulant

In an attempt to disclose whether the variations in prothrombin activity observed in rats on the two diets were dependent upon alterations of vitamin K concentration rather than on any inherent difference in response by the coagulation mechanism to the drug, each animal received chloromycetin* daily. It was hoped that the reduction of the intestinal flora brought about by the antibiotic would diminish the vitamin K synthesis in the gut. Each animal, therefore, was maintained on one of the diets for one week; during the second week 400 mg./kg. body weight chloromycetin was given daily in addition. On the fifteenth day 150 mg./kg. "tromexan" was administered and 24 hours later the prothrombin time was determined as before. It must be noted that throughout the experimental period each animal daily received the vitamin mixture which contained water-soluble vitamin K.

Results.—Following the administration of the anticoagulant to animals receiving antibiotics, both series manifested a more pronounced hypoprothrombinaemia than was observed when they were kept on the diets only. The differences between those on diets A and B were, however, still apparent. The average prothrombin time for animals on diet A was 24.0 sec., while that for those on diet B was 33.2 sec.

This difference between the results obtained in Groups 4 and 5 suggests that vitamin K plays an important role in the response to the anticoagulant (Payling Wright, 1952). When the diet is high in carbohydrate, moreover, the sterilizing effect of the chloromycetin is more efficient than when the fat content is high. On diet B the reduction of vitamin K was presumably greater; the response to the anticoagulant was consequently uninhibited, and a pronounced hypoprothrombinaemia ensued.

* "Paediatric Chloromycetin Palmitate," Parke, Davis and Co., Ltd.

Group 6: Effect of Raising Bile-salt Content of Gut on Response to Anticoagulant

Since one of the chief differences in the gut contents with diets of high- and low-fat constitution lies in the concentrations of available bile salts, it was possible that the discrepancies in prothrombin level observed between animals on the two diets might be dependent upon these steroids. With diet A it was possible that the bile salts might be fully utilized in ensuring fat digestion and that the full absorption of the anticoagulant might consequently be impaired. Such a condition might be rectified by the administration of bile salts before giving the anticoagulant.

After one week on the respective diets each rat daily received 100 mg./kg. bile salt in solution for a further week. On the fifteenth day 150 mg./kg. "tromexan" was given as before, and the prothrombin times were determined 24 hours later.

Results.—In this group the average prothrombin times for the animals on diets A and B were found to approximate more closely than in any of the other groups. The average time for those on diet A was 26.5 sec., while for those on diet B it was 28.6 sec. The presence of additional bile salts in the alimentary canal appeared to affect the response of animals on both diets. In this group the augmented response is therefore probably dependent upon an increased absorption from the gut, and the approximation of the findings in the two series suggests that those animals which receive a high-fat intake are less able to absorb the anticoagulant completely if the bile salt content is not augmented.

Comment

With the recognition of the importance of the absorption of vitamin K in modifying the response to oral anticoagulants of both man and experimental animals, it is strange that the circumstances which may alter conditions for its synthesis and absorption during anticoagulant therapy have not been more fully explored. Overman, Field, Baumann, and Link (1942) made suggestive and preliminary observations on rats, upon the effects of supplementing stock diets with alfalfa, fat, wheat-germ oil, choline, or vitamin C. They found that alfalfa, which is a rich source of vitamin K, was the only substance which altered the response of their animals to dicoumarol. In their experiments, however, the anticoagulant was mixed with the food so that animals which, for any reason, had a lowered appetite ingested less of the drug; the doses received by individual rats were thus

undetermined. Though these workers reported that "additions of fat and choline to the basal diets did not materially alter the degree of hypoprothrombinaemia," they suggested that the slightly raised resistance to dicoumarol that they noticed with choline and fat might be due to large amounts of these lipid substances causing retardation of absorption of the drug from the gut.

During the present series of experiments it became apparent that when the diet contained large quantities of fat the anticoagulant was less effective in producing hypoprothrombinaemia. This result is not wholly explicable by alterations in the amount of vitamin K available, for all our animals daily received a supplement of the water-soluble form of the vitamin that sufficed for normal health. Moreover, the suppression of the synthesis of vitamin K by chloromycetin failed to abolish the difference between the prothrombin responses in animals maintained on the two diets. The effects of diet thus appeared to be independent of the vitamin available. It may be noted, however, that when the vitamin K was reduced with chloromycetin the prothrombin times of all animals, irrespective of their diets, were considerably prolonged—an observation that confirms that of Irving Wright (1952) for patients who received simultaneous anticoagulant and antibiotic therapy.

To what dietetic influence, then, could the differences in prothrombin times observed in our experiments be attributed? From the findings in Group 6 it appears probable that the failure of the oral anticoagulants to produce a full response in animals results from its incomplete absorption. This partial failure is more evident in animals maintained on a high-fat intake, the high-lipid content of the gut retarding the absorption of the anticoagulant. When digestion of fats was improved by the oral administration of extra bile salts, the responses of the two series approximated, showing that the absorption of the "tromexan" in the presence of the two diets was now more nearly alike.

The problems posed in the introduction to this paper can only be partly answered by the results in the foregoing groups of experiments. First, the question whether differences in response to anticoagulants in patients undergoing treatment can be attributed to variations in the absorption of the drug from the gut. The results from animals that had received additional bile salts strongly suggest that the absorption factor may be of importance in determining the degree of response to a therapeutic dose of anticoagulant, for from our findings it appears probable that anticoagulant absorption may be impaired if the concentration of these steroids in the gut is too low. Secondly, the question whether the coagulation mechanism fails to respond to absorbed anticoagulant cannot be directly answered. It is possible that our findings that a high-fat diet lessens mortality for rats may be interpreted as being partly dependent upon the lipaemic condition of the blood, a condition which is accompanied by a decreased clotting time (Waldron *et al.*, 1951; Fullerton *et al.*, 1953). If the view of these two groups of workers that lipaemia entails an increased tendency to clot is admitted, this condition may be regarded as partially counteracting the effect of the anticoagulant; hence more of the drug is required to prolong the prothrombin time in the presence of lipaemia. Finally, the question of the importance of vitamin K in modifying the response to dicoumarol-like anticoagulants is again confirmed, though it is doubtful whether fluctuations in response can be attributed to variations in the absorption of this vitamin.

In trying to assess the reasons for fluctuations, such as are met with in clinical practice in the responses of patients to oral anticoagulants, it is essential to realize that several interacting factors may operate simultaneously. Variations in diet, which alter absorption conditions in the gut, may of themselves be insufficient to influence the degree of hypoprothrombinaemia produced by a given dose of anticoagulant, but when such a change is accompanied by alterations in the gut of such a kind as to diminish the available vitamin K a far more profound change from the expected normal may follow the administration of the drug. Though it is not yet possible to analyse the effects of dietary change or of the metabolism of the intestinal bacteria on the coagulation mechanism while the patient is under the influence of anticoagulants, it would appear advisable, from the foregoing experiments, to standardize within fairly narrow limits the fat intake of all cases receiving these drugs, and to avoid, whenever possible, their simultaneous administration with oral antibiotic or chemotherapeutic substances.

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BLOOD COPPER AND ITS RELATIONSHIP TO THE GLOBULINS

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Holmberg and Laurell (1948) gave the name "caeruloplasmin" to a blue copper globulin complex which they had isolated from both pig and human blood (1947). It was obtained by ammonium sulphate precipitation and was found to be active as an oxidase against p-phenylenediamine. They considered it to be an α globulin with a molecular weight of about 150,000. Other workers (Scheinberg and Gitlin, 1952; Bearn, 1953; Bearn and Kunkel, 1954a) agreed with these findings and also showed that caeruloplasmin was absent or very greatly reduced in hepatolenticular degeneration. Previously, however, Cohn (1948) had stated that copper in the human serum was combined with β globulin, and in 1949 Thompson and Watson had shown that 25% of the blood copper was bound to γ globulin, 55% to β globulin, and only 10% to α globulin. Holmberg and Laurell (1951) obtained good correlation between the levels of copper and of oxidase activity in blood in normal subjects, but this fact seems at variance with the observation that, while the copper in the serum of patients with hepatolenticular degeneration may be reduced by half, yet the oxidase activity may be almost nil.

This paper records the results of further experiments designed to elucidate these problems.

Material and Methods

Blood was obtained from normal subjects and from patients suffering from various disorders, including five cases of hepatolenticular degeneration, with adequate safeguards to avoid contamination with extraneous copper.

The globulins were separated from the serum by precipitation with 15%, 19.6%, and 26.8% anhydrous sodium sulphate saturation. Ether was used to facilitate separation, which was performed by centrifugation at 5,000 r.p.m. in a manner similar to that used by Thompson and Watson (1949). The following differences of technique were employed. The mixture of serum and salt was shaken by hand for five minutes in a water bath at 40° C., and the centrifuge cups were also warmed to this temperature. Copper was estimated in both globulin precipitate and in the remaining solution as a check on the results. The method of estimation of the copper was a modification of the Eden and Green (1940) technique as used previously (Cumings, 1951), which involves the precipitation of the proteins by trichloracetic acid.

The oxidase activity of the serum and of each of the globulin fractions was estimated in a manner identical with that used by Bearn and Kunkel (1954a). The benzidine technique of Holmberg and Laurell (1951) was also employed.

Results

Thompson and Watson quote the reasons for assuming that the method they employed does give a reliable basis for the separation of the three globulins. Globulin fractions were accordingly submitted to electrophoresis on paper so that an estimate of the accuracy of the results obtained could be determined. Fig. 1 shows one example of such an electrophoretic experiment. It shows that 15% salt saturation yielded a precipitate containing only γ globulin and that at 19.6% saturation as well as γ and β globulin there was a very slight trace of α_2 globulin. However, the amount of α_2 globulin was a very small fraction of the total α_2 present in the serum when the curve obtained at 26.8% precipitation is inspected. This latter curve shows that all the globulins are precipitated at 26.8% saturation. In this experiment no albumin was found, but in one only of the other experiments a trace of albumin was detected. Apart from this experiment all other electrophoretic curves were practically identical with the one shown here. It would seem therefore reasonable to use this technique as a method for separating the globulin fractions in serum.

In the following tables only the percentages of the total copper bound to each globulin are recorded. It was found in every experiment that the amount of copper in the precipitate and in the remaining solution when added together gave a yield

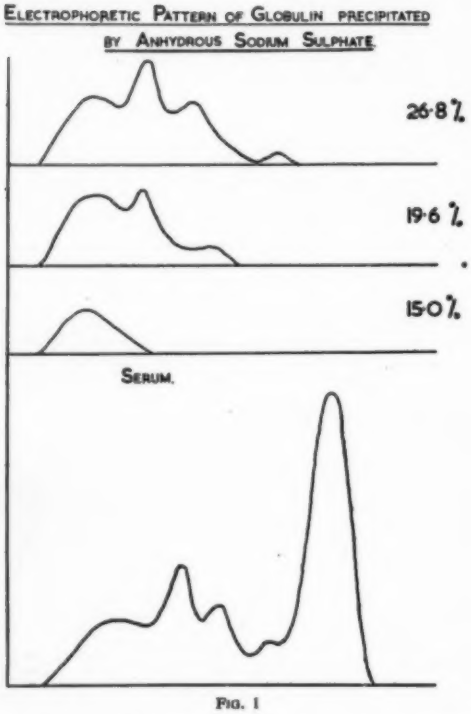

FIG. 1

of between 95% and 105% of the total serum copper.

Table I shows the results obtained in 10 normal subjects, from which it is seen that copper is bound in appreciable quantities by each globulin, and these findings are in agreement with those of Thompson and Watson, even though there is a greater variation from one specimen of blood to another in our experiments.

TABLE I
GLOBULIN COPPER LEVELS IN NORMAL ADULT SUBJECTS

Case	Total Serum Copper (μg./100 ml.)	Percentage of Total Copper Recovered from Globulin		
		γ	β	α
1	102	33	33	18
2	90	30	45	15
3	115	22	34	39
4	175	36	29	35
5	122	21	46	29
6	123	6	47	42
7	150	22	48	25
8	127	6	23	55
9	180	9	52	35
10	143	9	66	17
Average ..	132·7	19·4	42·3	31·0

Table II gives the results found in one patient with multilobular cirrhosis of the liver, in one patient with jaundice, the result of impacted gall stones, and in four patients with involuntary movements akin to those seen in hepatolenticular degeneration. These last four patients showed neither increased copper nor amino-acid excretion in the urine, and in no case was the diagnosis of hepatolenticular degeneration entertained. Included in the table are the protein contents of serum and of globulin fractions.

TABLE II
GLOBULIN COPPER LEVELS IN VARIOUS DISORDERS

Case	Total Serum Copper Level (μg./100 ml.)	Percentage of Total Copper Recovered from Globulin		
		γ	β	α
Jaundice (gallstones) ..	160 (6·0)	15 (1·2)	66 (1·2)	16 (0·6)
Cirrhosis of liver ..	193 (6·45)	32 (1·5)	32 (1·35)	19 (1·05)
Abnormal movements with syphilis ..	97 (6·7)	12 (0·5)	48 (1·5)	36 (1·2)
Abnormal movements with multiple sclerosis	113 (7·5)	19 (0·6)	60 (1·2)	16 (0·9)
Athetosis	103 (6·6)	35 (0·4)	41 (0·8)	10 (0·9)
Choreo-athetosis ..	137 (7·7)	26 (0·7)	44 (1·5)	14 (0·9)

( ) Indicates protein content in g./100 ml.

The results are almost identical with those seen in Table I as far as the copper is concerned, and it is further evident that the amount of globulin present bore no relationship to the amount of copper.

If all the above 16 results are added to the 12 obtained by Thompson and Watson the mean amounts of copper bound to γ, β, and α globulin are 23%, 48%, and 20% respectively.

Five cases of hepatolenticular degeneration were also examined by this method, and the findings are recorded in Table III. All show striking differences from the normal pattern, for all except one show an almost complete absence of copper on the α globulin, while the fifth case showed an absence of copper on the β globulin. All five cases had a low serum copper level, but none of the five showed

TABLE III
GLOBULIN COPPER LEVELS IN HEPATOLENTICULAR DEGENERATION

Case	Total Serum Copper (μg./100 ml.)	Percentage of Total Copper Recovered from Globulin		
		γ	β	α
18550/49	66 (6·0)	10 (1·05)	58 (0·9)	1 (0·75)
F.K.	57 (5·6)	29 (0·8)	11 (1·5)	2 (0·3)
J.B.	64 (6·4)	19 (0·4)	25 (1·1)	7 (0·7)
„ 3 months later ..	53	24	26	4
P.B.	50 (7·4)	11	14	5
21191/50	60 (6·1)	25 (0·3)	8 (0·8)	26 (0·8)
„ 3 months later	62	15	4	28

( ) Indicates protein content in g./100 ml.

any abnormal protein pattern. A further abnormal feature is that between 30% and 60% of the serum copper appears firmly attached to the albumin, as was found by estimating the copper in the remaining solutions after precipitation of all the globulins with the 26.8% salt. A second examination was possible in two of the cases at some weeks' interval after the original estimations, and each pair of results are almost identical, as can be seen in the table.

It might be mentioned that the first and last cases are two of the four cases described previously (Cumings, 1951).

Table IV shows the results of measuring oxidase activity of three normal sera and of that of the globulin fractions from these same cases using the method of Bearn and Kunkel. The blood was obtained by therapeutic venesection, and this enabled us to obtain an adequate amount.

TABLE IV
OXIDASE ACTIVITY OF GLOBULIN FRACTIONS IN CONTROL SUBJECTS

	Case 1			Case 2			Case 3		
Serum copper ..	127 μg./100 ml.			180 μg./100 ml.			143 μg./100 ml.		
Oxidase activity of serum	158 $\mu 10_2$/ml./hour			167 $\mu 10_2$/ml./hour			119 $\mu 10_2$/ml./hour		
Globulin fractions ..	γ	β	α	γ	β	α	γ	β	α
% Copper estimated	6	23	55	9	52	35	9	66	17
% of total oxidase activity	23	77	0	22	65	13	9	91	0

The percentage amounts of oxidase activity of the fractions is given, but the actual amounts of oxygen uptake of all three fractions approximated very closely to the total figure of the serum. It is seen that β globulin shows the greatest activity, and that in the first and the third cases no activity was found in the α fractions. The electrophoretic charts shown in Fig. 1 were obtained from the serum and the globulin fractions actually used for oxidase activity estimations in Case 3, and, as the bulk of the α globulin is only present in the 26.8% salt saturation, this should have yielded oxidase activity had there been any in this fraction. A small amount only of oxidase activity was found in the γ globulin fraction.

The examination of the sera from the five cases of hepatolenticular degeneration showed either no oxidase activity or only a very small amount, whether examined by the benzidine method or by the method using p-phenylenediamine, as can be seen in Table V. Further, in one of these cases no oxidase activity was found in any of the three globulin fractions.

TABLE V
OXIDASE ACTIVITY OF SERUM IN HEPATOLENTICULAR DEGENERATION

	18550/49	F.K.	J.B.	P.B.	21191/50
Serum copper in μg./100 ml.	66	57	64	50	60
Oxidase activity of serum in $\mu 10_2$/ml./hour	0	0	0	0	5·4
	Control	Control	F.K.	J.B.	21191/50
Extinction readings using benzidine technique	0·08	0·078	0	0·02	0·019

Sera from two normal subjects has been mixed with sera from two cases of hepatolenticular degeneration, and oxidase activity determined in the mixtures. No inhibitory effect by the pathological sera was found in either case.

Discussion

The results obtained in normal sera are in agreement with those of Thompson and Watson, and show that there is a definite distribution of copper between all three globulins, but that the larger amount of copper is normally attached to β globulin. Although it may perhaps be said that the methods used do not perfectly separate each of these three globulins, yet the bulk of evidence quoted by Thompson and Watson, taken with our electrophoretic evidence, does suggest that this method may reasonably be used. This view is further supported by the results of oxidase activity of the different globulins. Other workers have only used ammonium sulphate precipitation of all globulins to obtain the copper globulin complex. The electrophoretic studies of Bearn and Kunkel (1954b) cannot be used to refute this, as they were examining sera only some six or 12 hours after giving isotope copper, for Earl, Moulton, and Selverstone (1954) have shown conclusively that after giving Cu_{64} the albumin fraction takes up nearly 80% of this copper, and that even after 24 hours 20% or more is still attached to the albumin. In fact there is a transference of the Cu_{64} from albumin to globulin only after 24 hours, and therefore the studies of Bearn and Kunkel do not reflect the normal state of copper distribution.

Holmberg and Laurell suggest that there is a direct relationship between increased blood copper levels and increased oxidase activity, and by this they can only mean that all the copper in the serum possesses this enzymic activity. Does it mean then that each copper globulin complex has such activity? This would not seem to be true from the results shown in Table IV. Here the

greatest oxidase activity was in the β globulin fraction, and in two cases none was found in the α fraction even though some copper was found in this fraction in these cases. So far as we have found there has not been an absolutely good parallel between copper levels and oxidase activity in normal sera, but a very large number of such examinations are necessary.

Even greater difficulties emerge when the cases of hepatolenticular degeneration are considered. Serum copper levels are reduced in some cases, being half the normal, yet the oxidase activity is reduced even more. This has also been the experience of Bearn and Kunkel. Examination of the globulin fractions has shown an absence of α globulin copper in four cases, but the γ and β globulin copper complexes showed no oxidase activity. Further, one case differed from the others in twice showing no β globulin copper. It must be admitted that at first it was felt that a mistake might have been made, even though it was known that this was virtually impossible. However, after passing a renal calculus this patient returned to hospital and a second examination was possible, and this verified the initial results. There appears to be no clinical distinction between this case and the others. Further, two of these patients had not been treated in any way before examination.

There is another point of importance that has to be considered. A large portion of the serum copper in these five cases was attached to the albumin fraction, and it is very probable that this is inactive in so far as oxidase activity is concerned. The copper remaining to the globulins may then be either too small in amount to exert oxidase activity, or it may be that a certain minimal amount is required before it can show such activity. It seems unlikely that there is an antagonist present to prevent such activity. The presence of copper combined with albumin as found in the above experiments is in accord with the results shown by Bearn and Kunkel (1954a), when they examined serum from cases of hepatolenticular degeneration electrophoretically on starch blocks. This may be of considerable importance, for it would seem that this albumin copper might be easily transferred to the tissues and it may be passed through the glomerulus with the urine. An attempt was made to see if it were possible that this copper was attached not to albumin but to an oligopeptide, but so far we have not been successful. Should this have been true it would have fitted in very well with the suggestions made by Uzman (1953) that the urinary copper is so linked.

Zimdahl, Hyman, and Cook (1953) showed that in hepatolenticular degeneration there is an abnormal uptake of copper from the intestinal tract, and should this be correct then this absorbed copper may be linked with albumin in the same way as Cu_{64} is in the experimental work of Earl *et al.* It may then be available to be deposited in the tissues or excreted in the urine before it has had time to be transferred from the albumin to the globulins, or because of some specific action which prevents such a transfer, or because of the absence of some enzyme which normally acts by transferring copper from albumin to globulin.

Summary

The amount of copper bound to γ, β, and α globulins has been estimated in normal subjects and in some pathological states, including five cases of hepatolenticular degeneration. It has been shown that β globulin binds most copper, followed by the γ and α fractions.

The cases of hepatolenticular degeneration differ in that in four cases no α-binding copper was found, while in the remaining case no β globulin copper was present.

Oxidase activity was found to be absent in all five cases of hepatolenticular degeneration, and also absent in each of the globulin fractions in one case.

Oxidase activity of the three globulin fractions was measured in three control subjects, and the greatest amount was found in β globulin, while no activity at all was detected in the α globulin fraction in two cases.

These findings are briefly discussed in relation to the nature of hepatolenticular degeneration.

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ISOLATION AND EXAMINATION OF URINARY METABOLITES CONTAINING AN AROMATIC SYSTEM

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It is becoming increasingly evident that pathological states may lead to urinary excretion of abnormal metabolites, and that in such cases identification of these metabolites may be a valuable diagnostic aid and may give a clue to the fundamental lesion. Classical test-tube colour reactions are of little value owing to the enormous complexity of urine and the, in general, relatively unspecific nature of the reagents. The exceedingly high resolving power of chromatographic methods and their technical simplicity enables separation of metabolites to be carried out before identifying tests are applied and so makes it possible to derive far more information from much less material than was formerly possible.

The method described here allows qualitative examination of one group of urinary metabolites, those containing an aromatic, e.g., benzene or indole, ring by a rapid and simple procedure.

Experimental Procedure

Principle of the Method.—The aromatic urinary metabolites are adsorbed on deactivated charcoal, eluted with aqueous phenol, the eluate concentrated, fractionated on paper chromatograms, and suitable spray reagents applied.

Preparation of Deactivated Charcoal.—A charcoal deactivated with 4% by weight of stearic acid is convenient for routine use. To a solution of 16 g. stearic acid in 1,200 ml. ethanol is added slowly with stirring 400 g. charcoal (e.g., B.D.H. activated). The mixture is left one hour with occasional stirring, and then 10 l. of water is added slowly with stirring. After settling, the bulk of the supernatant is drawn off, and the deactivated charcoal filtered on a Buchner funnel, washed with a large quantity of distilled water, and air-dried.

Phenol for Elution.—"Liquid" phenol (phenol liquefactum, B.P.), 80 ml., is diluted to 1 litre with water.

Treatment of Urine.—The urine is acidified with acetic acid and centrifuged. Deactivated charcoal is added to the supernatant and the mixture shaken occasionally for about 5 min. The charcoal is filtered off on a small Buchner funnel and washed with water to remove the remaining salts, urea, sugars, and aliphatic amino-acids. It is then eluted by slowly passing the phenol solution through it, followed by more water. The eluate is evaporated in a water-bath under reduced pressure. The phenol is eliminated by steam-distillation during this process. To ensure complete removal of the phenol further water can be added and the mixture again concentrated. The concentrate thus obtained is applied directly to paper chromatograms.

For routine use about 100 to 200 ml. of urine is suitable, but there is no difficulty in using as little as 5 ml. The amounts of charcoal and phenol to be used vary greatly because of variations in the metabolite content of the urine, but a suitable amount can easily be estimated after some experience has been gained. If sufficient charcoal has been used the urine filtrate after the initial adsorption will be almost or quite colourless. If too much has been used a large volume of phenol will have to be passed through the charcoal before any colour is eluted, as the phenol molecules will first adsorb on vacant spaces on the charcoal before displacing metabolite molecules already present. Phenol should be passed till the eluate is almost colourless, followed by distilled water to remove eluted material still in the body of the charcoal. As a rough guide, for 200 ml. of normal urine about 2 to 3 g. deactivated charcoal and 50 to 70 ml. of the aqueous phenol should be suitable.

If the phenol eluate is allowed to stand before evaporation, a precipitate of uric acid may settle out. If of no interest to the experiment this is best discarded. Similarly on concentration of the eluate uric acid separates. Its presence in the concentrate does not affect the chromatography.

Paper Chromatography.—For routine examinations one-dimensional chromatograms in butanol:acetic acid:water (4:1:5 v/v/v; Partridge, 1946) have been found most suitable. R_F values given in Table I refer to this solvent, using Whatman No. 1 paper and the descending technique. R_F values have been determined at room temperature without any attempt to standardize conditions, and are not therefore to be taken as more than an indication of the approximate position on the chromatogram. Chromatograms run under such unstandardized conditions are adequate for almost all purposes provided

reference standards are run simultaneously. R_F values can be modified, amongst other things, by a metabolite being displaced by other substances in a crowded region of the chromatogram, and by variations in the *p*H of the applied solution. For example, the R_F value of a base, such as adrenaline, may be different if applied as free base and as hydrochloride. In such cases a reference standard should be applied in the appropriate ionic form.

Detection of Metabolites.—The following reagents and methods are of particular value :

Fluorescence under Ultra-violet Light.—When examined in ultra-violet light which has passed through a Wood's glass filter many metabolites are revealed as characteristically coloured (see Table I) fluorescent spots. Ultra-violet light is also the most convenient way of revealing the solvent front. Normal urine contains traces of many unidentified fluorescent substances, some of which may be of plant origin (from the diet) rather than true metabolites.

Ninhydrin.—Ninhydrin is of value chiefly for the aromatic amino-acids and also, using a modified reagent and the resulting ultra-violet fluorescence (Jepson and Stevens, 1953), for tryptamine derivatives.

Ehrlich's Reagent.—This is of particular value for detecting (*a*) indole derivatives, (*b*) pyrrole derivatives, and (*c*) aromatic amines. It is convenient to have two reagents available, differing in acid strength, which will be referred to as the " normal " and " strong " Ehrlich's reagent respectively. The " normal " reagent is a 2% (w/v) solution of *p*-dimethylaminobenzaldehyde in approx 1.3 N HCl (concentrated HCl diluted with 6 parts of water). The " strong " reagent is a 2% (w/v) solution of the aldehyde in approx. 6 N HCl (3 parts concentrated HCl to 2 parts water). After spraying, the papers are allowed to dry at room temperature.

Indole and pyrrole derivatives in general give shades of red or blue, and the colours may change characteristically with time. Aromatic amines give a yellow colour, which may be modified to, e.g., orange by suitable neighbouring groups (cf. kynurenine, hydroxyanthranilic acid). Aliphatic amines may give a weak yellow after some time. Urea gives a bright yellow (hence an important reason for removing it). Indican, though an indole derivative, gives a brown colour ; the compound formed in this case is chemically related to indigo, and is of a different type from the compounds usually formed with indole derivatives (for further discussion of indican see below).

The use of two reagents differing in acid strength is advantageous, as the coloured compounds formed are indicators. At the *p*H of the " normal " reagent aromatic amines give a yellow colour immediately, whereas coloured compounds from indole derivatives may take some time to form. At the *p*H of the " strong " reagent derivatives of aromatic amines are formed but remain colourless, whereas indole derivatives, including indican, give a colour rapidly. It is thus possible to distinguish the two types of compound even if present in the same spot. Ultimately the picture obtained with both reagents is the same, the indole compounds appearing slowly with the " normal " reagent and the aromatic amines appearing as the HCl evaporates from the paper after the " strong " reagent.

Pauly's Reagent.—The paper is sprayed with diazotized sulphanilic acid. Certain phenols develop a colour at this stage, whereas the majority only do so after a subsequent spray with alkali (aqueous Na_2CO_3 or ethanolic ammonia). Colours obtained are listed in Table I. Urinary phenols have been studied especially by Boscott, Bickel, and their colleagues, and reference should be made to their publications (e.g., Boscott and Bickel, 1953, 1954 ; Boscott, 1954). Pauly's reagent also detects glyoxaline derivatives, e.g., histamine, histidine, and urocanic acid. It is possible that the stabilized diazo compounds now being developed for the van den Bergh reaction will prove a useful substitute for diazotized sulphanilic acid when they are more readily available.

Ekman's (1948) *Reagent.*—Aromatic amines are diazotized either by spraying the paper with acidified nitrite or by exposing the damped paper to nitrous fumes, and coloured azo compounds are then formed by spraying with an ethanolic solution of 1-ethylaminonaphthalene. Much additional information can be obtained in the first stage (spraying with acidified nitrite) (cf. Dalgliesh, 1952a). Yellows or browns are given by *o*-aminophenols (due to benzoxdiazole formation) and indole derivatives (due to N-nitroso compounds). A blue (due to indigo) is given by indican. Pink (urorosein reaction) is given by indoleacetic and indoleaceturic acids and also by indole itself.

Ammoniacal Silver Nitrate.—The papers are sprayed and allowed to dry at room temperature. The reagent is of especial value in detecting substances containing readily oxidizable groups such as *o*-aminophenol or catechol derivatives (cf. Dalgliesh, 1952a ; Dalgliesh and Tekman, 1954). These rapidly give black or brown-black spots. Many other substances will react, but more slowly.

Other Reagents.—For particular compounds or types of compound many other reagents are available. Some useful examples are the Altman reagent (Gaffney, Schreier, Di Ferrante, and Altman, 1954), which is the only simple way of detecting hippuric acid on chromatograms, and is also useful for substituted hippuric acids (e.g., *o*- and *p*-aminohippuric acids). In the author's experience it is more convenient to allow colours with this reagent to form at room temperature, rather than by heating. James' ferricyanide reagent (James, 1948 ; James and Kilbey, 1950), which is particularly useful for the catechol amines ; the urorosein reaction (see under Ekman's reagent above) ; for phenols the Folin-Ciocalteau reagent (the commercially available reagent diluted 1 in 4), and the reagent of Barton, Evans, and Gardner (1952) ; for nicotinic acid derivatives and related quaternary pyridinium compounds see Kodicek and Reddi (1951) and Holman (1954) ; for the Thormählen reaction (for melanogens) applied to paper see Leonhardi (1953).

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF SOME SUBSTANCES ENCOUNTERED IN URINE

Substance	R_F[1]	Fluorescence[2]	Ehrlich's Reagent[3]	Pauly's Reagent[4]	Ekman's Reagent[6] and Urorosein Test[9]	Other Reagents for Confirmatory Tests
Adrenaline	0·39	—[12]	—	Salmon-pink	—	Ammoniacal silver nitrate, James' ferricyanide reagent
p-Aminobenzoic acid ..	0·79	—	(n) Immediate yellow	—	Magenta	[10]
p-Aminosalicylic acid	0·84	—	(n) ,,	Brown	,,	[10]
o-Aminohippuric acid ..	0·76	Purple	(n) ,,	—	,,	Altman reagent (pink)
p-Aminohippuric acid ..	0·63	—	(n) ,,	—	,,	Altman reagent (orange-pink)
Anthranilic acid ..	0·90	Purple	(n) ,,	—	,,	[10]
Anthranilic acid glucuronide	0·52	Purple	(n) ,,	—	,,	[10]
2: 5-Dihydroxyphenylalanine	0·32[8]	—	—	Brown-pink	—	Ammoniacal silver nitrate; James' ferricyanide reagent; ninhydrin
3: 4-Dihydroxyphenylalanine	0·28	—	—	Magenta	—	Ammoniacal silver nitrate: James' ferricyanide reagent; ninhydrin
3: 4-Dihydroxyphenylethylamine	0·44	—	—	,,	—	Ammoniacal silver nitrate : James' ferricyanide reagent
Hippuric acid	0·85	—	—	—	—	Altman reagent (orange)
Homogentisic acid ..	0·80	—	—	Brown-pink	—	Ammoniacal silver nitrate
3-Hydroxyanthranilic acid	0·84	Blue-purple	(n) Yellow or yellow-pink	Pink	(Yellow-brown)	Ammoniacal silver nitrate
5-Hydroxyanthranilic acid	0·54	Strong pale blue	(n) Immediate yellow	Brown	Blue (appears slowly)	Ammoniacal silver nitrate
m-Hydroxybenzoic acid	0·88	—	—	Orange	—	
p-Hydroxybenzoic acid	0·88	—	—	,,	—	
5-Hydroxyindoleacetic acid	0·75	—	(s) Blue	Brown-pink before alk., intensifying afterwards	(Brown, or red if strong acid)	Ammoniacal silver nitrate
3-Hydroxykynurenine	0·31[7 8]	Green	(n) Pink or orange-pink	Pink-red	(Yellow-brown)	Ammoniacal silver nitrate
5-Hydroxytryptamine	0·33	—	(s) Grey-blue→grey purple→green	Brick-red	,,	Modified ninhydrin: ammoniacal silver nitrate
5-Hydroxytryptophan	0·17	—	(s) Grey-blue→blue-purple→green	Red-magenta	,,	Ninhydrin: ammoniacal silver nitrate
Indican	0·41	—	(s) Brown	Slow brown to pink[11]	(Blue developing slowly)	(See text)
Indole	0·93	—	(s) Carmine	—[5]	(Pink)	
Indoleacetic acid ..	0·90	—	(s) Magenta→blue	—[5]	,,	
Indoleaceturic acid ..	0·82	—	(s) ,,	—[5]	,,	
Indolepropionic acid ..	0·91	—	(s) Blue-magenta→blue	—[5]	(Yellow-brown)	
Kynurenic acid ..	0·60	Weak blue	—	—	—	
Kynurenine	0·37[8]	Strong pale blue	(n) Orange	—	Magenta	Ninhydrin[10]
Noradrenaline ..	0·30	—[12]	--	Salmon-pink	—	Ammoniacal silver nitrate: James' ferricyanide reagent
Phenylalanine ..	0·55	—	—	—	—	Ninhydrin
Porphobilinogen ..	0·49	—	(s) Magenta→weaker blue	—	—	
Riboflavin	0·21[7]	Yellow	—	—	—	
Salicylic acid	0·91	Purple	—	Orange-yellow	—	
Salicyluric acid ..	0·83	Blue-purple	—	Yellow	—	
Skatole	0·94	—	(s) Violet→blue	—[5]	(Yellow-brown)	
Tryptamine	0·60	—	(s) Red→violet→grey-violet	—[5]	,,	Modified ninhydrin
Tryptophan	0·45	—	(s) Violet→green	—[5]	,,	Ninhydrin
Tyrosine	0·40	—	—	Pink	—	,,
Tyramine	0·57	—	—	,,	—	,,
Urea	0·50	—	(n) Immediate bright yellow	—	—	
Xanthurenic acid ..	0·60	Blue	—	Brick-red	—	

[1] On Whatman No. 1 paper, descending technique, using butanol : acetic acid : water, 4: 1: 5 v/v/v. See comments in text.

[2] In ultra-violet light which has been passed through a Wood's glass filter. Substances showing a very weak fluorescence are not noted, as this would almost certainly be masked in a chromatogram of a urine extract.

[3] (n) = normal reagent and (s) = strong reagent. For differences in the rates of appearances of colour with the two reagents see text. The reagent indicated is the most convenient for identification.

[4] Colours after the alkaline spray, unless otherwise stated.

[5] If the reagent contains an excess of nitrite the colours entered in brackets under Ekman's reagent may also be observed.

[6] The colours in brackets represent colours visible after diazotization (urorosein and related reactions. See text and note 9).

[7] If present in appreciable amount will be visible as a yellow spot on the untreated chromatogram.

[8] If D- and L-forms are present these may be resolved into their optical isomers (see Dalgliesh, 1952b).

[9] If it is the urorosein reaction which is of interest more intense colours are obtained by spraying with approx. 6N HCl to which sodium nitrite solution has been added.

[10] These substances may show some degree of yellow with the Altman reagent.

[11] This is complicated by the ready oxidation of indoxyl formed on hydrolysis of the indican. Especially in presence of excess nitrite only a blue (indigo) spot may be obtained.

[12] Fluorescent oxidation products may be observed.

Other methods which can give useful information are to elute a substance from the chromatogram and compare its behaviour before and after hydrolysis; to determine the migration on paper electrophoresis at varying *p*H values; and to determine the ultra-violet absorption spectrum (e.g., Dalgliesh, 1952a).

Results

Table I summarizes the reactions of many compounds encountered in urine examinations. Various points of interest of some of these compounds are noted below.

Drugs and Drug Metabolites.—It is desirable to withdraw all drugs for 24, or preferably 48, hours before urine collection. If this is not possible, allowance should be made for drug metabolites which might be present. Many of the popular drugs and their metabolites are readily isolated from urine and detected by the present method. For example, sulphonamides can produce urines so rich in metabolites as to obscure the normal constituents. Aspirin gives rise to salicyluric acid (see below), the excretion of which can last for a considerable time after the dose. *iso*Nicotinic acid hydrazide, *p*-aminosalicylic acid, and chloromycetin are other examples of drugs giving rise to readily detectable metabolites which might obscure the normal metabolic picture. Conversely, of course, the method is useful for studying the metabolism of suitable drugs.

Anthranilic acid is excreted in congenital hypoplastic anaemia (Altman and Miller, 1953).

3-Hydroxyanthranilic acid may be excreted in acute tuberculosis (e.g., Musajo, Spada, and Coppini, 1952; Makino, Satoh, Fujiki, and Kawaguchi, 1952).

5-Hydroxyindoleacetic acid is a normal constituent of human urine (Titus and Udenfriend, 1954) and is probably derived from tryptophan by way of 5-hydroxytryptamine. The excretion may be increased in certain types of cancer (Clerc-Bory, Pachéco, and Mentzer, 1954).

3-Hydroxykynurenine may be excreted, sometimes with kynurenine, in many cases of fever of varying origin (Dalgliesh and Tekman, 1954).

5-Hydroxytryptamine (enteramine, serotonin) probably does not occur in urine but may be encountered in other body fluids, e.g., blood, or tissue extracts (cf. Dalgliesh, Toh, and Work, 1953; for detection see this paper and Jepson and Stevens, 1953).

5-Hydroxytryptophan is a normal urinary constituent (Titus and Udenfriend, 1954).

Indican.—Much controversy has occurred in the past as to whether or not indican is a normal constituent of urine. In the author's experience it is always present in a fresh normal urine. Negative results previously reported have probably been due to insufficiently sensitive tests. The present methods are far more sensitive than, for example, the frequently used Obermayer test. The indicator properties of the compound formed between indoxyl (from hydrolysis of the indican) and Ehrlich's reagent allow a test sensitive to a fraction of a microgram. The normal brown is allowed to develop on the chromatogram and the paper is then sprayed with saturated sodium acetate. The brown changes to an intense cherry-red, whereas the colour given by most other substances with Ehrlich's reagent disappears. Indican sometimes appears as multiple spots, but, as authentic potassium indoxyl sulphate added to urine can behave in the same way, these multiple spots are probably due to the one substance. If large amounts of indican are present the untreated chromatograms may show fast-running pink and blue spots. These are due to indirubin and indigo respectively, formed by oxidation of indoxyl.

Indole and skatole do not normally occur in urine but may be encountered if there has been faecal contamination.

Indoleacetic and Indoleaceturic Acids.—Indoleacetic acid is a normal urinary constituent. The excretion of indoleaceturic acid may be related to the metabolic defects in phenylketonuria (Armstrong and Robinson, 1954).

Noradrenaline.—The presence of abnormal amounts in the urine may allow diagnosis of phaeochromocytoma when other methods fail (von Euler, Lund, Olsson, and Sandblom, 1953).

Riboflavin somewhat resembles hydroxykynurenine in its fluorescence, but is readily distinguished by other tests.

Salicyluric acid is frequently encountered due to the widespread use of aspirin. In R_F value and fluorescence it resembles hydroxyanthranilic acid but is readily distinguished by other tests.

Xanthurenic Acid.—This may be separated from kynurenic acid on chromatograms run with inorganic salt solutions, e.g., the 5% sodium formate or 20% KCl solutions used by Boscott (1952).

Not all types of compounds which are probably extractable from urine by the present method have been examined. It is likely that the method could, for example, be extended to some of the steroids, and to purines and pyrimidines and their metabolites. As uric acid is easily isolated by this method it is probable that xanthine would be too; xanthinuria (Dent and Philpot, 1954) should there-

fore be readily detectable. It is hoped in the future to investigate some of these possibilities.

Quantitative Aspects of Metabolite Recovery.—The recovery of many substances has been checked at various times both from pure solution and for substances added to urine. In no case has quantitative recovery been observed, but in the majority of cases recoveries have been in the region of 60 to 90%. Such a recovery is quite adequate for qualitative work. Lower recoveries have been observed for porphobilinogen (35–40%) and for certain hydroxylated metabolites, e.g., 5-hydroxytryptamine, where recoveries are variable but on the whole unsatisfactory. Methods for improving the recovery of such substances are being examined.

Results with two substances are recorded in Tables II and III.

TABLE II
RECOVERY OF TRYPTOPHAN BY CHARCOAL ADSORPTION PROCEDURE

Weight of Tryptophan Taken	Percentage Recovery		
	(a)	(b)	(c)
125 μg.	65	73	62
250 „	71	68	69
1,250 „	88	84	84
2,500 „	80	—	—
10 mg. ..	90	84	89
30 „	87	78	78
50 „	78	69	64
100 „	48	—	—

Column (a), tryptophan dissolved in 10 ml., column (b), 20 ml., column (c), 50 ml. of solution.

TABLE III
RECOVERY OF ANTHRANILIC ACID BY CHARCOAL PROCEDURE

Weight of Anthranilic Acid Taken	Percentage Recovery
380 μg.	95
1·7 mg.	85
3·7 „	92·5
17·7 „	88·5
45·0 „	90

Tryptophan Recovery.—The stated amount of tryptophan was dissolved in water (10 ml., 20 ml., or 50 ml.; solution was effected with acid if necessary) and each solution treated by the above procedure with 500 mg. deactivated charcoal, followed by elution with 35 ml. of aqueous phenol. The phenol was removed by concentration as described above, the eluate made up to a known volume, and the tryptophan estimated by the procedure of Horn and Jones (1945).

It will be seen from the table that a satisfactory recovery (taken as greater than 50%) is obtainable over a range of concentration greater than 4,000-fold. This is an important advantage when working with urine, where metabolites may be present in widely differing concentrations. The figures also suggest that small amounts of a substance such as tryptophan could be satisfactorily detected. For example, if 200 ml. of urine is assumed to be available and tryptophan is excreted at the rate of 1 mg./day, at least 50 μg. should be present in the urine concentrate, which would give a detectable level of tryptophan even if divided between several chromatograms. In the experiments referred to in Table II tryptophan was observed in the filtrate from the charcoal adsorption when 30, 50, or 100 mg. of tryptophan was used. From this one can deduce that for isolation purposes a level of about 20 mg. tryptophan/500 mg. deactivated charcoal would be most advantageous.

Anthranilic Acid Recovery.—A similar procedure was used, the anthranilic acid being dissolved in 25 ml. of water before charcoal treatment. Recovery was estimated by the Bratton and Marshall (1939) procedure as used by Knox (1953). The results are shown in Table III.

Discussion

The method is based on the work of Synge and Tiselius (1949), who found that on deactivated charcoal columns the elution of aromatic metabolites depended on the number of aromatic systems rather than on other functional groups present. Thus compounds with one aromatic system tended to be eluted together, and were quite separate from aliphatic compounds or compounds with two aromatic systems. The present technique is essentially a simplified form of displacement analysis. The most active positions of the charcoal, on which metabolites would be adsorbed irreversibly, are blocked with stearic acid. This deactivated charcoal can then adsorb aromatic metabolites reversibly. On treatment with a more strongly adsorbed substance the metabolite molecules are displaced and emerge in the eluate. Phenol is an excellent displacing agent, as it is eliminated from the eluate by steam distillation during concentration.

The method as described above has proved most valuable for routine urine examinations (e.g. Dalgliesh, 1952a ; Charconnet-Harding, Dalgliesh, and Neuberger, 1953 ; Dalgliesh and Tekman, 1954). As can be seen from the figures quoted, the recoveries are not quantitative. The work of, for example, Hagdahl, Williams, and Tiselius (1952) and Porath and Li (1954) suggests that if column methods were used conditions might well be found for the isolation of a specific aromatic substance with a high recovery. But such conditions would vary for each substance, and the method described here,

though not quantitative, gives satisfactory qualitative recoveries for a wide range of metabolites. An unsatisfactory recovery is normally due to the metabolite being incompletely eluted, adsorption being in general complete. Simple alterations which might be found advantageous for isolation of a particular metabolite are: (*a*) Altering the degree by which the active positions of the charcoal are blocked, e.g., by using charcoal deactivated with 6% or 8% by weight of stearic acid. A metabolite which is strongly adsorbed is more easily removed from a charcoal in which only weakly active areas are available for adsorption. (*b*) The *p*H of the eluant may be altered, e.g., by using acidified phenol (though hydrolysis of conjugates may then occur during concentration). (*c*) A displacing agent other than phenol may be used, provided it can be removed from the eluate without also removing the metabolites under investigation.

The advantages of the method are the following: (1) It is cheap, simple, and rapid. The actual working time in preparing a sample for chromatography is well under half an hour, and the total time, if an efficient water pump is used for the evaporation, need be no more than an hour. (2) A high degree of concentration is readily obtained, and interfering substances, such as salts, urea, sugars, and aliphatic amino-acids, are eliminated. (3) The method is far simpler (and quicker) than solvent extraction procedures. The latter are frequently accompanied by difficulties due to emulsion formation. In general the urine must first be hydrolysed, as many metabolites are excreted mostly or entirely as hydrophilic conjugates which cannot be directly extracted. Moreover, hydrolysis can very largely destroy indolic compounds. By the charcoal adsorption procedure conjugates are isolated unchanged and the conjugation pattern can be readily assessed. This can be of considerable interest, e.g., as a guide to liver function, and is of course also highly desirable in, e.g., studies of the metabolic fate of drugs. (4) The method is far simpler and shorter than electrolytic desalting procedures. The latter result in loss of strongly charged metabolites, and the conditions are sufficiently vigorous to cause chemical changes in labile molecules. Moreover urea, which can cause interference in paper chromatography, is not removed. The charcoal method is largely carried out at neutral *p*H under mild conditions and isolates charged and uncharged molecules equally, with elimination of both salts and urea. (5) Ion-exchange resins, like charcoal, can be used to isolate a given type of metabolite, in this case based on net charge. Suitable combinations of both methods, allowing separation of aromatic metabolites with a given type of charge, can be most useful in isolation studies.

The disadvantages of the method are: (1) It is not quantitative. (2) If a metabolite is more strongly adsorbed than the eluant it will not be eluted to any appreciable extent, and will remain undetected.

Work attempting to overcome these disadvantages is at present in progress. In spite of them the method is considered to be of great value and worthy of wider use. It reveals, even in normal urine, a large number of unidentified substances. The identification of these and of metabolites abnormally excreted in pathological states offers a rich field for research.

Summary

A method is described for rapid isolation of the group of urinary metabolites containing a simple aromatic system. The method is applicable both to studies of metabolic defects, e.g., of the aromatic amino-acids, and to studies of drug metabolism.

The chromatographic behaviour of many substances likely to be encountered in urine is summarized.

I thank Miss R. Paul and Mr. A. Asatoor for skilled assistance, and also the many people who have given me authentic compounds.

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J. clin. Path. (1955), **8**, 79.

A SIMPLIFIED METHOD OF INVESTIGATING STEATORRHOEA

BY

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For years, the five- or ten-day fat-balance study has been considered the only sure way to determine the presence of steatorrhoea. Owing to the shortage of trained nursing staff and diet kitchens in many hospitals, this method of investigation is open to many inaccuracies. To physicians, the method has seemed impracticable and time-consuming, and pathologists have not welcomed the laborious analyses of fat in dried faeces. Anderson, Frazer, French, Gerrard, Sammons, and Smellie (1952) have for some years used a quicker method of fat analysis based on the principles of homogenizing the wet faeces and estimating the total fatty acid content of a sample by the method of van de Kamer, ten Bokkel Huinink, and Weijers (1949). This technique has been used here, and, following the work of Annegers, our patients have not been on a weighed intake of fat but on normal ward diet. Annegers, Boutwell, and Ivy (1948) carried out investigations on 40 normals who were given standard amounts of fat in their diet of two types, namely, lard and homogenized vegetable oil. Specimens were collected for five-day periods and the fat content determined. Their conclusions were that fat balance "digestibility" studies are of questionable validity, since within ordinary dietary limits faecal fat excretion is, in their experience, independent of dietary intake.

Procedure

The basic simplifications of our method are: (1) Patients are on the usual ward diet. (2) The total fat excretion over only three consecutive days is measured. (If the scatter of observations is great a further three days' samples are obtained.)

In the ward the patient's 24-hour output of faeces is collected in a container which is then sealed, labelled, dated, and despatched to the laboratory, and this procedure is repeated for two successive days. If, for a day, the patient is constipated, the collections are continued until four days of output have been completed. The results are expressed as a mean 24-hourly fat excretion over a given period, usually three days, or as a mean three-day fat excretion calculated as fatty acid according to Goiffon (1942).

Method

The specimen is then homogenized according to the method of Anderson *et al.* (1952). The whole of the 24-hour specimen is placed in an "ato-mix" mixing machine (graduated at 900 ml.). Distilled water is added until the consistency allows the machine to run at half-speed in order to remove air. It is allowed to run until the air is removed, and then the volume made up to 900 ml. The machine is then allowed to run at full-speed for one minute. The mixed faeces are allowed to stand for a few minutes so that the small air bubbles may rise to the top. The fat content of a sample of this is estimated by van Kamer's method.

Results

Investigations were performed on 11 normal cases and on 11 cases of mild steatorrhoea developing after the Polya type of gastrectomy. The results are shown in Figs. 1 and 2.

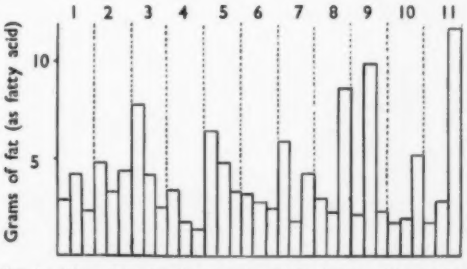

FIG. 1.—Mean three-day fat excretion 11.72±3.17 g. of fat (as fatty acid) in normals. Mean daily fat excretion 3.91±2.45 g. of fat (as fatty acid) in normals.

It will be seen that normal people excrete less than 5 g. of fat per day over a three-day period, and the scatter of daily values is much less than in

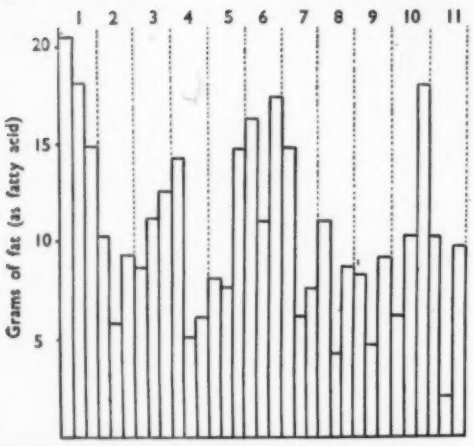

FIG. 2.—Mean three-day fat excretion 30.87±11.41 g. of fat (as fatty acid) in cases of steatorrhoea. Mean daily fat excretion 10.29±4.49 g. of fat (as fatty acid) in cases of steatorrhoea.

those with steatorrhoea. If we assume that 100 g. of fat per day is as much or more than is contained in the normal ward diet, then an excretion of 5 g. of fat per day corresponds to 95% absorption, the usually accepted lower level of normal absorption. Thus 5 g. of fat per day can safely be accepted as the upper limit of the normal range of excretion. Using these criteria and this method it will be seen from the charts that the distinction between controls and cases of steatorrhoea is quite clear cut. It is a method where any error due to variations in fat intake would tend to "iron out" differences between the normal and abnormal groups, so the clear-cut distinction between the two groups is all the more significant.

Summary

A simplified method, which is useful in cases of steatorrhoea and where there is no necessity to use a balanced fat diet, is described.

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A METHOD FOR THE DETERMINATION OF GLUTAMINE IN CEREBROSPINAL FLUID AND THE RESULTS IN HEPATIC COMA

BY

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The free amino-acids normally present in cerebrospinal fluid are leucine, valine, alanine, glycine, serine, and glutamine. The concentration of each amino-acid is much less than that found in plasma, with the exception of glutamine, which is in higher concentration in the cerebrospinal fluid. Walshe (1951) examined the cerebrospinal fluid of three patients in hepatic coma by paper chromatography and found an excess of glutamine. This important observation suggested that if a simple method for the quantitative determination of the glutamine content of the cerebrospinal fluid could be developed, such an estimation might prove valuable in the differential diagnosis of cases of hepatic coma.

Material

Hepatic Coma. — Cerebrospinal fluids were obtained from seven patients in hepatic coma. Brief clinical histories follow and the more usual liver function tests are shown in the Table.

Case 1.—A boy aged 11 had acute necrosis of the liver following an attack of infective hepatitis nine weeks before. A period of coma lasting for 48 hours occurred 12 days before death. He recovered consciousness, and his general condition improved following energetic treatment with electrolyte solutions and glutamic acid, but he died suddenly 10 days later. Necropsy was performed.

Case 2.—A man aged 31 had cirrhosis of the liver following an attack of infective hepatitis three years before. He had signs of portal hypertension with oesophageal varices. He was admitted to hospital following repeated haematemeses and became disorientated and violent. He lapsed into deep coma for four days but recovered following treatment with glutamic acid, and is in fair health and at work 18 months later.

Case 3.—A boy aged 12 had glycogen disease with secondary cirrhosis of the liver (biopsy performed). He had been in and out of hospital on several occasions for investigation and paracentesis abdominis. While being nursed at home he became disorientated and restless and was readmitted to hospital in a stuporose condition. He died in deep coma four days later.

Case 4.—A woman aged 32 had acute necrosis of the liver following a fulminating attack of infective hepatitis three weeks before. She became restless, incoherent, and then alternated between violent delirium and stupor. Finally she lapsed into deep coma and died two days later. Necropsy was performed.

Case 5.—A woman aged 27 had acute necrosis of the liver following an attack of infective hepatitis three months before. She also had a history of jaundice at the age of 9. During the terminal stage of the illness she became disorientated and lapsed in and out of coma. Necropsy was performed.

Case 6.—A woman aged 63 had cirrhosis of the liver of unknown aetiology. She had a history of several attacks of coma with moderate health between attacks, but no history of haematemesis but frequent epistaxes and blood loss from haemorrhoids. She was admitted to hospital in a confused state and later became comatose. She recovered sufficiently to leave hospital but died six months later. Necropsy was performed.

Case 7.—A man aged 60 had cirrhosis of the liver with terminal mesenteric thrombosis. He had been in hospital on several occasions for paracentesis abdominis and was finally readmitted on account of abdominal pain and vomiting. He became comatose and died. Necropsy was performed.

Cirrhosis of the Liver.—Cerebrospinal fluids were obtained from nine cases of cirrhosis of the liver with evidence of liver damage. The more conventional liver function tests are shown in the Table.

Control Cases.—Cerebrospinal fluids were obtained from 31 patients in whom no disease of the liver was suspected or found. Five patients were in coma caused by non-hepatic conditions, five were suffering from meningitis, three from haemat-

TABLE
RESULTS OF LIVER FUNCTION TESTS AND CEREBROSPINAL FLUID GLUTAMINE LEVELS IN HEPATIC DISEASE

Case No.	Sex	Age (Years)	Serum Bilirubin (mg./100 ml.)	Thymol Turbidity (Units/100 ml.)	Alkaline Phosphatase (K.A. Units/100 ml.)	Serum Proteins (g./100 ml.)			C.S.F. Glutamine (mg./100 ml.)
						Total	Albumin	Globulin	
Hepatic Coma									
1	M	11	17·0	18	14	6·4	1·3	5·1	54
2	M	31	1·5	8	17	5·1	2·1	3·0	35
3	M	12	7·2	2	56	6·0	1·1	4·9	54
4	F	32	39·0	5	9	7·5	4·4	3·1	30
5	F	27	9·0	3	21	8·1	2·6	5·5	30
6	F	63	3·4	18	27	5·0	2·2	2·8	43
7	M	60	3·5	5	7·8	7·1	3·3	3·8	33
Cirrhosis of the Liver									
8	F	42	6·4	18	6·5	6·1	2·3	3·8	21
9	M	72	9·5	7	30	5·5	1·4	4·1	27
10	M	77	1·8	—	20	5·0	2·2	2·8	20
11	F	45	2·3	5	8	5·8	2·0	3·8	23
12	M	61	1·5	4	11	5·5	1·7	3·8	16
13	M	61	2·6	9	9	7·6	3·5	4·1	22

emesis due to peptic ulceration, and the remaining 18 from conditions in which lumbar puncture was a justifiable investigation.

Methods

No special precautions were taken in the collection of the cerebrospinal fluids. Analysis was usually performed on the day of collection, otherwise the fluid was frozen and kept in an ice chamber.

Chromatography.—The one-dimensional, circular paper technique of Giri and Rao (1952) has been used. For successful chromatography of the amino-acids in cerebrospinal fluid the fluid must be electrolyte- and protein-free. This is accomplished most simply by using a cation ion-exchange resin as described in a preliminary communication by Prior and Whitehead (1953).

The resin (Zeo Karb 225) is activated by mixing with twice its volume of 10 w/v% hydrochloric acid and allowing to stand for approximately two hours. The resin is then washed free of acid with distilled water and placed in a column 15 cm. × 0.5 cm., when 1 ml. of cerebrospinal fluid is passed through the column and 15 ml. of water used to wash it through. The resin retains cations and amino-acids; anions and protein pass through the column unchanged. The amino-acids are then displaced from the column by 15 ml. of N/1 ammonia solution, the cations remaining on the column. The first 3 ml. of eluate is discarded, the next 12 ml. is collected and evaporated under reduced pressure at 37° C. The dried residue is redissolved in 1 ml. of distilled water and the resulting solution used for chromatography. (Two amino-acids, which are not normally encountered in cerebrospinal fluid, are lost in this technique; taurine is not retained on the column, arginine needs a stronger ammonia solution for elution.) For chromatography 20 c.mm. (2 × 10 c.mm.) of the resulting solution is used. The solvent used is a butyl alcohol:acetic acid:water mixture. The papers are developed in the usual way with ninhydrin.

Assay of Glutamine.—Glutamine is completely hydrolysed to glutamic acid and ammonia by dilute acid at 100° C. The only other substance encountered in cerebrospinal fluid that produces ammonia under these conditions is urea. Other amino-acids, asparagine, protein, and glucosamine, even in relatively large amounts, are not hydrolysed. We have used acid hydrolysis to ammonia as a method of assaying the glutamine content of cerebrospinal fluid.

To 1 ml. of cerebrospinal fluid in a 12 ml. test tube, 0.2 ml. of 10 v/v% sulphuric acid is added. The tube is placed in a boiling water bath for exactly 10 minutes, removed, and cooled in cold water, when 0.3 ml. of 10 w/v% sodium hydroxide is added followed by 5 ml. of water, 0.5 ml. of 2% gum ghatti solution, 2 ml. of Nessler's reagent, and the colour immediately read in a colorimeter using a blue-green filter. A standard solution of glutamine (25 mg./100 ml.) is similarly treated. A blank determination performed in exactly the same manner but omitting the boiling may be performed, but this is unnecessary if the fluid is examined on the day of collection. The standard glutamine solution should be prepared on the day of analysis. Urea is partially hydrolysed under these conditions, and therefore the urea concentration of the fluid is also determined and a correction made. The hydrolysis of urea at concentrations up to 400 mg./100 ml. is 1%, so that the correction is urea concentration (mg./100 ml.) $\times \frac{5}{100}$ mg. "glutamine"/100 ml.* The correction is usually less than 2 mg./100 ml. The highest urea concentration encountered in this investigation was 73 mg./100 ml., entailing a correction of 3.6 mg./100 ml. in a fluid containing 57 mg. glutamine/100 ml.

* Each mg. of urea yields $\frac{28}{60 \times 100} = 0.0047$ mg. of nitrogen. One mg. of glutamine yields $\frac{14}{146} = 0.096$ mg. of nitrogen, so that the factor for urea correction is $\frac{0.0047}{0.096} = 0.05 \left(\text{or } \frac{5}{100}\right)$.

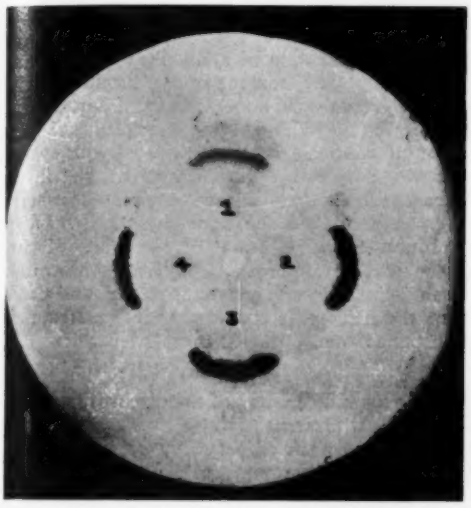

FIG. 1.—Paper chromatography of cerebrospinal fluids from normal control (1), hepatic comas (2) and (3), and cirrhosis of the liver (4).

Results

Chromatography.—With the technique described above, using 20 c.mm. of cerebrospinal fluid for chromatography, normal fluids usually show one band, that due to glutamine. To demonstrate the presence of other amino-acids larger quantities of fluids are required. The purpose of this investigation was to compare the glutamine concentration of the fluids, and 20 c.mm. was used throughout the investigation. Fig. 1 illustrates typical results from normal fluids (1), fluids from patients in hepatic coma (2 and 3), and fluid from a patient with cirrhosis of the liver (4). In each chromatogram the very prominent band is glutamine. This was confirmed by control runs with glutamine and in some cases by two-dimensional chromatography.

Determination of Glutamine.—Fig. 2 shows the glutamine concentrations found in 18 normal fluids and in 13 fluids from patients suffering from diseases in which disturbance of liver function was not suspected. The range is from 6 mg./100 ml. to 14 mg./100 ml. glutamine with the exception of one case of haemophilus meningitis, where the glutamine content was 18 mg./100 ml.; fluids from such cases have shown a pronounced increase in several amino-acids.

The glutamine concentration in nine cases of cirrhosis of the liver ranged from 16 mg./100 ml. to 31 mg./100 ml., and in seven cases of hepatic coma from 30 mg./100 ml. to 54 mg./100 ml.

In Cases 1, 2, and 6 the glutamine content of the cerebrospinal fluid was determined during a period of coma and when the patient was conscious.

Case No.	Diagnosis	Glutamine (mg./100 ml.)	
		Conscious	Comatose
1	Infective hepatitis	31	54
2	Cirrhosis of liver	21	35
6	" " "	23	43

In all cases there was close agreement between the chromatographic appearance of the glutamine band and the level determined by acid hydrolysis.

Discussion

These results indicate that in cirrhosis of the liver there is an increased concentration of glutamine in the cerebrospinal fluid, and during hepatic coma this is further increased. A control series of fluids from patients in coma other than hepatic and from patients suffering from miscellaneous conditions has been analysed, and the glutamine level is not altered with the exception of the fluid from a patient suffering from haemophilus meningitis, when a slightly raised level was found. We suggest therefore that determination of the glutamine content in the cerebrospinal fluid, which is simple to perform and speedy, may be of value in the differential diagnosis of hepatic coma. Murphy, Chalmers, Eckhardt, and Davidson (1948)

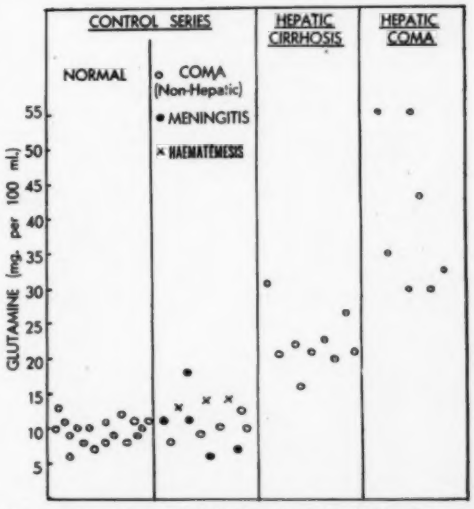

FIG. 2.—The cerebrospinal fluid glutamine values in hepatic coma, cirrhosis of the liver, and the control series.

studied 40 patients in hepatic coma and concluded that, apart from evidence of severe hepatic failure, there was no distinctive physical sign or biochemical estimation characteristic of hepatic coma, and furthermore the biochemical findings were not significantly different in the pre-comatose stage of the disease. Our results in three cases suggest that the onset of coma is associated with a significant rise in the cerebrospinal fluid glutamine, and, in the only case in which we had an opportunity of repeating the estimation after complete recovery from coma, the glutamine level fell to a figure within the range for non-comatose cases of cirrhosis of the liver (Case 2).

The role of glutamine in the cerebrospinal fluid of patients suffering from cirrhosis of the liver and hepatic coma has not yet been elucidated. Krebs (1935) investigated the metabolism of ammonia in brain tissue and showed that it combined with glutamic acid to form glutamine. Weil-Malherbe (1950) reviewed the metabolism of glutamic acid by nervous tissue and concluded that the primary function of glutamic acid was its ammonia-binding mechanism. Several recent investigations have suggested an association between raised blood ammonia levels and the development of coma in patients suffering from disease of the liver. Gabuzda, Phillips, and Davidson (1952) showed that clinical changes indistinguishable from those encountered in hepatic coma can be induced by feeding ammonium salts to patients with cirrhosis of the liver, and McDermott and Adams (1954) described stuporose attacks in a patient with a portal-caval fistula which were associated with a demonstrable rise in blood ammonia. Riddell and McDermott (1954) thought that most cases of hepatic coma were caused by ammonia intoxication and found some correlation between blood ammonia levels and neurological symptoms. On the other hand, Singh, Barclay, and Cooke (1954) could find no correlation between blood ammonia levels and the clinical state. The technical difficulties of measuring blood ammonia levels probably accounts for some discrepancy. The finding of an increased amount of glutamine in the cerebrospinal fluid in hepatic coma lends support to the suggestion that an increased concentration of ammonia in the cerebral tissues may play some part in producing this type of coma. Ajmone-Marsan, Fuortes, and Marossero (1949) thought that the pharmacological action of ammonia on the central nervous system was excitatory rather than depressant, and in the present investigation the stage of coma was ushered in by a period of confusion and excitement in five out of seven patients. Further study of glutamine levels in cerebrospinal fluid may throw light on the glutamic acid/glutamine system and determine the place of glutamic acid in the treatment of hepatic coma.

Summary

The glutamine content of the cerebrospinal fluid of patients with cirrhosis of the liver and in hepatic coma has been investigated by chromatography and by a simple assay method.

The glutamine level in cerebrospinal fluid is raised in cirrhosis and still further raised in hepatic coma. The determination of cerebrospinal fluid glutamine is advocated as a biochemical aid in the differential diagnosis of hepatic coma.

The possible relationship between ammonia intoxication and an increased cerebrospinal fluid glutamine level is discussed.

Some cases of hepatic coma were under the care of other physicians, and we wish to thank them for their co-operation in this investigation, and also Drs. A. P. Prior and J. M. Walshe for their interest and encouragement.

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TECHNICAL METHOD

A SIMPLE METHOD FOR THE ESTIMATION OF PEPSIN IN GASTRIC JUICE

BY

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(RECEIVED FOR PUBLICATION APRIL 8, 1954)

The albumin method of estimating pepsin is slow and crude, while the more modern haemoglobin methods (Anson and Mirsky, 1932 ; Bucher, Grossman, and Ivy, 1945) are too technical for the average laboratory. Apart from its simplicity, the method described below has the advantage that the sample of gastric juice is diluted sufficiently to obviate the action of pepsin inhibitors in the juice (Bucher and Beazell, 1941). The method is particularly suitable for the determination of low values of pepsin concentration.

Principle

Pepsin changes edestin into edeston. A saturated solution of sodium chloride produces an opalescence when mixed with a solution of edestin but not with a solution of edeston.

Stock Solutions

Stock Pepsin Solution.—Pepsin, 50 mg., is dissolved in 100 ml. N/10 HCl., and kept in the ice chest.

Stock Edestin Solution.—Edestin, 0.5 g., is dissolved in a mixture of 150 ml. N/10 HCl and 350 ml. distilled water by warming and stirring. The solution is filtered and kept in the ice chest.

Stock pepsin and edestin solutions should be used only when fresh.

Also required are saturated NaCl and N/10 HCl.

Method

To 1.0 ml. stock edestin solution 1.8 ml. N/10 HCl and 0.2 ml. stock pepsin solution are added and the mixture is incubated at 37° C. At intervals of two minutes a few drops of the mixture are withdrawn into a saturated solution of NaCl. The absence of opalescence means that digestion of edestin to edeston is complete. The time taken for complete digestion, usually 10 to 15 minutes, is noted.

The above procedure is repeated with the following alterations : (*a*) Test gastric juice, 0.2 ml., is used in place of the stock pepsin solution and filtered. (*b*) Incubation is for *exactly* the time required for complete digestion by the stock pepsin solution. (*c*) When incubation is complete 3 ml. saturated NaCl solution is added and mixed. The opalescence is compared with that of standard opalescent tubes.

STANDARD OPALESCENT TUBES*

	100% Edestin	75% Edestin	50% Edestin	25% Edestin	12·5% Edestin
Stock edestin solution (ml.)	1·00	0·75	0·50	0·25	1·125
Saturated NaCl (ml.)	2·0	2·0	2·0	2·0	2·0
N/10 HCl	Add sufficient to make volume up to 4·0 ml.				

* These can, more conveniently, be made artificially.

The amount of pepsin in the test solution can be easily calculated. It can be expressed either indirectly in terms of the amount of edestin digested or else directly in mg.% ; e.g., suppose the test gastric juice digested only 50% of the given amount of edestin as the same volume of stock pepsin solution, then the gastric juice would contain half the concentration of pepsin as the latter, viz., 25 mg.%.

If the test solution digests completely in less time than the stock pepsin solution, the unknown pepsin concentration can be calculated by repeating the test after first diluting the gastric juice.

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J. clin. Path. (1955), **8**, 86.

BOOK REVIEWS

Laboratory Aids in Endocrine Diagnosis. By Roberto F. Escamilla. (Pp. xii+131; 21 plates. 34s.) Oxford: Blackwell Scientific Publications. 1954.

This little book, which is almost encyclopaedic in scope, contains lists of laboratory tests applicable to the presently acknowledged endocrinopathies. Laboratory tests are given a wide application, including electrocardiograms, radiographs, the metabolic rate as well as those more strictly allied to clinical and chemical pathology. The final chapter, instead of the customary index, contains a recapitulation of the laboratory data collected under the headings of the various endocrine diseases.

Only the methods of the more complicated tests are given. These include, for example, two methods for blood glucose, vaginal smear techniques, and urine pregnancy tests. Although cancer diagnosis by vaginal smears grew out of work on endocrine studies it seems out of place in this sort of book to include a large colour plate (Fig. 11) depicting cervical cell pathology. It is interesting to find the Hogben pregnancy test relegated to the place of an "also ran," being displaced by male frog or toad tests. The chapter on testicular biopsy is well up to present-day teaching, but the few photomicrographs are technically ill chosen.

The book might be found useful for reference, but the author insists that laboratory aids do not replace an adequate clinical appraisal of a patient. Few would agree to repose confidence in one specially trained technician in interpreting results of some of the more complicated tests for clinical use. M. HAINES.

Experimental Diabetes and its Relation to the Clinical Disease. A Symposium organized by the Council for International Organizations of Medical Sciences established under the joint auspices of U.N.E.S.C.O. and W.H.O. (Pp. ix+352. 35s.) Oxford: Blackwell Scientific Publications. 1954.

It is still a matter of some surprise to some observers that the problem of human diabetes was not more or less finally solved by the studies which began with the demonstration by v. Herring and Minkowski that pancreatectomy was followed by diabetes and which culminated in the successful isolation in 1922 of insulin by Banting and Best. Indeed, the spate of work which followed this last discovery has presented far more problems than it has solved. The role of the pituitary and adrenal hormones is no nearer being understood. Even the important discovery of the diabetogenic action of alloxan has done nothing to tell us the cause of human diabetes, which remains as elusive as it was before the first experimental pancreatectomy. The literature in this field is so vast that any account attempting to bring together within a reasonable compass our knowledge of the subject is especially welcome.

In 1952 the Council for International Organizations of Medical Sciences sponsored a symposium on experimental diabetes and its relation to the clinical disease. The book *Experimental Diabetes* which records the papers presented at that symposium, together with the numerous discussions these papers provoked, provides a compendium invaluable to the biochemist, physiologist, or clinician who is concerned with diabetes. An excellent account by Best of recent work on the islets of Langerhans is followed by two papers by de Duve and by Candela on glucagon. The possible mechanism of the diabetogenic action of alloxan in the experimental animals receives particular attention, but the view that alloxan or uric acid may fulfil a physiological function in carbohydrate metabolism or be of importance in the causation of human disease is unconvincing. Two papers are concerned with the effects of adrenal steroids in carbohydrate metabolism; growth hormone and its relation to general metabolism, to diabetes, and to insulin also receive consideration. There are also contributions on the action of insulin as well as on its absorption, as well as papers discussing diabetes and heredity, diabetes and pregnancy, and the various clinical forms of the disease.

This book, which is astonishingly readable despite the large mass of factual information, is a praiseworthy attempt to bring together the views of clinicians and scientists, and may well stimulate a closer collaboration between these two groups of workers.

C. H. GRAY.

Porphyrins. Their Biological and Chemical Importance. By A. Vannotti; translated by C. Rimington. (Pp. 258; 15 plates. 50s.) London: Hilger and Watts. 1954.

Professor Vannotti's monograph "Porphyrine und Porphyrinkrankheiten" was published in 1937. Its value is indicated by the fact that so eminent an authority as Professor Rimington should have felt it desirable himself to translate it and thereby make it more readily available to English-speaking readers. This is not a translation solely of the 1937 monograph, as Professor Vannotti has specially made considerable additions to cover advances since that time. The recent literature is, however, less well covered. The manuscript was finished some time ago, but publication was unfortunately delayed. The considerable recent advances in our knowledge of porphobilinogen and porphyrin biosynthesis are thus not adequately covered.

The section on the chemistry of the prophyrins is principally confined to a statement of structures. Spectroscopic characteristics are well summarized with useful plates, and this is followed by a chapter on extraction and determination. The classical methods are well reviewed, but the paper-chromatographic and electrophoretic techniques now yielding most useful results in this field receive scant mention. The occurrence of porphyrins in nature is then considered.

The greater part of the book consists of a review of porphyrin metabolism in man in normal and pathological

states. The various porphyrias are well surveyed, with chapters on possible modes of treatment and on the pharmacological effects of porphyrins. The approach is often personal and considerable illustrative use is made of cases which have come under Professor Vannotti's own observation.

The weakness of the work lies in its arrangement. The new material covering recent work has been added, usually at the end of chapters, without integrating it with existing material. It is thus possible to find outmoded theories in one part of a chapter and more recent work refuting it later on with no cross-reference (e.g., Fischer's theory that the more carboxylated porphyrins are detoxication products is stated without qualification on p. 7 and refuted in a brief discussion of recent isotopic work only on p. 31). It is thus unwise to make use of isolated sections; chapters must be considered as a whole. A few footnotes by the translator help, but they are not adequate to replace the desirable greater integration of the text or, if this were impracticable, at least more adequate cross-references.

The translator has been careful not to superimpose his own views and has provided a most faithful translation. It is perhaps excessively loyal to the original to maintain some of the Continental usages, such as lactoflavin for riboflavin.

One of the most valuable features of the book is the extensive bibliography. One of the weaker features is the somewhat inadequate index. But the work as a whole remains a most valuable prime source for those interested in porphyrin metabolism.

C. E. DALGLIESH.

The Distribution in Human Blood Groups. By A. E. Mourant; with Foreword by H. J. Fleure. (Pp. xxi+438. 42s.) Oxford: Blackwell Scientific Publications. 1954.

It is less than 40 years since the Hirszfelds discovered that the frequency of ABO blood groups varied in different nations. This discovery started off an enormous amount of work on the distribution, first of the ABO blood groups, but soon also on that of the MN groups and later still particularly that of the Rhesus blood groups.

This book is the first which is a completely comprehensive survey of the data widely scattered in the literature. It complements the work on the genetics of the human blood groups, *Blood Groups in Man* by Race and Sanger, which has recently appeared in its second edition. Nevertheless there is a succinct and useful survey of blood group inheritance and it is elaborated to show the anthropological significance of the different blood group systems: ABO, Rhesus, MNS, Lewis, P, Lutheran, Kell, Duffy, Kidd. Other simple genetical systems such as the sickle cell trait or the ability to taste phenylthiocarbamide are rightly promoted to "honorary blood groups" and are fully discussed. Detailed surveys of geographical distribution are dealt with in separate chapters: northern and central Europe, Africa south of the Sahara, the Mediterranean area, Asia, Indonesia, Australasia, and America. There are chapters on hybrid and migrant populations, on the blood grouping of animals, and of bones and tissues ("blood group archaeology"). All this amounts only to the first half of the book and is summarized in a brilliant chapter, "Attempt at a Synthesis," which is headed appropriately by a quotation from Walter de la Mare:

> "Strange prodigy is man. Of so short stay,
> Yet linked with Vega and with Nineveh.
> Time—space: what matters it how far away,
> In this strange hall of mirrors through which
> we stray?"

Recently evidence has been forthcoming that simple genetical characters such as the ABO groups or the sickle cell trait may have a selective value protecting against some diseases and causing others. As the balance between advantages and disadvantages will depend on surroundings we may obtain here a new insight in the formation of the human races. It is of particular interest that the distribution of some blood group systems such as Rhesus and MNS is fairly uniform over whole continents, whereas the ABO blood groups show wide variations within comparatively small areas.

The second half of the book contains chapters on laboratory procedures and calculation of gene frequencies. A detailed bibliography of some 1,700 references is then presented which alone will make this book an outstanding work of reference. There follow maps of the world and of Europe showing the distribution of various blood group genes on the principle of isobars. This manner of presentation is quite new and produces fascinating surveys at a glance. This is followed by 26 blood group frequency tables and an exhaustive list of the frequencies of the sickle cell trait. At the end there are three indices, one topographical, one zoological to the bibliography, and one general to the text.

This scholarly work will be a useful guide for all pathologists to a special aspect of blood group serology —at present not yet of practical importance but already intriguing and theoretically fundamental. It will be an indispensable work of reference to those who are specialists in the field.

H. LEHMANN.

The Laboratory Diagnosis of Leptospirosis. By J. W. Wolff. (Pp. xiv+98; 5 illustrations. 27s. 6d.) Springfield, Ill., U.S.A. Charles C. Thomas, Oxford; England: Blackwell Scientific Publications, Ltd. 1954.

The methods, for diagnosing leptospiral infection by laboratory means, which are described in this book have been used for the very extensive work which has been done in Amsterdam during the last quarter of a century. During all this time Dr. Wolff has been one of the leaders in applying them to leptospiral diseases in Indonesia and in the Netherlands. The separate aspects of the practical work are clearly laid out in full detail: microscopical demonstration of leptospires in dark-ground preparations, in stained films and stained sections; culture, animal inoculation, and serological tests. The

pitfalls of identification are shown as in the risk of confusion in dark-ground preparations of protoplasmic threads for leptospires; also, most sensitive methods are described like the use of careful centifugation of the plasma from oxalated blood to concentrate leptospires for microscopical examination. In serology the author uses living cultures for the observation of agglutination and lysis by active serum, but he states that the end-point of agglutination of killed leptospires (which is the method usually adopted in Great Britain) is practically as high as the end-point of lysis of living organisms. In this country the formalin used to kill leptospires is made less harmful to the antigen by adding pyridin to it (Burke, V. (1933), *Amer. J. Path.*, **9**, 915).

Dr. Wolff indicates the proper method of examination for the different stages of a leptospiral infection and gives a synopsis and table of the very valuable work on serological differentiation of 36 types of leptospires by himself and Dr. J. C. Broom which was published recently. There are also brief accounts of the epidemiology of the infections caused by 24 types. There is a good index. This is a clearly written, authoritative, practical handbook on a group of infections of man and animals about which knowledge is rapidly expanding throughout the world.

J. M. ALSTON.

Biochemical Investigations in Diagnosis and Treatment. By John D. N. Nabarro. (Pp. x+299; illustrated. 25s.) London: H. K. Lewis.

The rapid development of chemical pathology in recent years has opened up a very wide field in which both physicians and clinical pathologists need guidance if full advantage is to be taken of recent discoveries in diagnosis and treatment of disease. Dr. Nabarro's book sets out to clarify these developments from the point of view of the young physician.

The many subjects covered comprise water and electrolyte metabolism, calcium and phosphorus metabolism, protein and nitrogen, carbohydrate and fat metabolism, diseases of the gastro-intestinal tract and of the liver, investigation of renal function, the cerebrospinal fluid, the endocrine glands, vitamins and poisons, with an extensive series of tables of normal values of blood, urinary and faecal constituents.

The matter comprised in the book is well up to date, but treatment of different subjects is uneven: for instance, the chapters on electrolyte metabolism and on the endocrine glands contain much information which does not occur in the older books, and should be very helpful both to the physician and the general pathologist.

On the other hand, much of the chapter on carbohydrate metabolism is old "textbook stuff" which is familiar ground to the majority of readers: the two rare conditions, Von Gierke's disease and galactosaemia, mentioned in this chapter should receive fuller treatment if the article is to be of assistance to the clinical pathologist, and it would be helpful if selected references could be given to important work on such conditions: as it is, all four references at the end of the carbohydrate chapter refer to general textbooks or monographs on diabetes.

The arrangement of the book where the first seven chapters deal with the metabolism of different groups of substances and the remainder of the book largely with the biochemical changes met with in diseases of various organs, necessarily leads to a good deal of duplication. The Fanconi syndrome, for instance, is referred to in six different places, yet it is difficult anywhere to get a comprehensive idea of what is meant by the Fanconi syndrome. The index is also twofold, dealing with, first, an index of investigations, and, secondly, an index of conditions: one complete index would probably be more convenient.

Details of laboratory tests are not given, except in cases where it is necessary for the physician to collect suitable specimens, where instructions as to collection are given. However, in some cases the instructions are insufficiently precise: for example, in the pyruvate tolerance test (p. 242) it is mentioned that blood should be transferred to ice-cold trichloro-acetic acid as quickly as possible—but how much blood and how much acid are not specified: either instructions as to blood taking should be omitted entirely or should be complete.

The series of tables of normal values divided into those "usually undertaken in hospital laboratories" and those "not usually undertaken" should be very useful for reference.

The main criticism is that this book falls between the two stools of the new and the old: a decision has to be made whether this is to be a general elementary textbook, in which elementary discussions of all types of biochemical investigation are included, or an introduction to recent advances in chemical pathology, in which case much of the elementary matter already covered in other books could be omitted and greater attention given to more recent work. In this latter case, more references might well be included.

E. N. ALLOTT.

At the meeting of the Joint Committee of the Association of Clinical Pathologists and Association of Clinical Biochemists, it was reported that vials of freeze-dried serum suitable for biochemical investigation will be ready for distribution some time in April. This material has been prepared by Messrs. Glaxo, and an analysis has been carried out by members of the Joint Committee. A mean of the analyses will be included with each sample on a separate piece of paper. It will be distributed by Messrs. David Keeler, Ltd., of 39, Wigmore Street, London, W.1, price 4s. 6d. per vial, postage extra. This is the actual cost price of the material.

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HISTOLOGICAL DIFFERENTIATION BETWEEN PHAEOCHROMOCYTOMA AND MELANOMA OF THE SUPRARENAL GLAND

BY

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Two patients presented with suprarenal tumours of similar macroscopic appearance and with some histological features in common. The final diagnosis in each case, however, was different; one was a typical phaeochromocytoma, while the other was believed to be a primary melanoma of the suprarenal. This paper illustrates the pathology of the two tumours, showing the difficulties encountered in their differentiation. The clinical histories of the two patients are of great interest and are described fully by Slessor *et al.* (1955) and Slessor and MacLeod (1955).

First Case Report

J. A., a man aged 49 years, presented with a history of episodes of shivering, diarrhoea, and vomiting: a large tumour was felt above the left kidney. The histamine test and blood assays for adrenaline were positive. At operation on May 24, 1951, a large tumour was removed from the site of the left suprarenal gland. The patient recovered satisfactorily but, on re-testing with histamine, a positive response was still obtained. He was able to return to work and remained symptom-free and normotensive but was kept under observation. Eighteen months later a mass was found in the left side of the neck. This mass, with attached thyroid tissue, was removed at operation on October 22, 1953. Again, the patient recovered satisfactorily and is now back at work.

The abdominal tumour was smooth and ovoid, measuring $12 \times 10 \times 10$ cm. and weighing 410 g. (Fig. 1). It contained cystic spaces of varying sizes with dark brown grumous fluid. The solid parts consisted of soft, greyish pink tissue with many small areas of old and recent haemorrhage which gave a reddish brown appearance to the whole mass. A portion of the tumour was sent for biological assay and was found to contain 1.75 mg. adrenaline and 10.75 mg. noradrenaline per g. of fresh tumour.

Histologically, the tumour was surrounded by a thick, dense fibrous capsule through which tumour cells did not penetrate at any point. Clusters of refractile yellowish to dark brown pigment granules, presumably in crushed macrophages, were present in the inner layers. Outside the capsule, compressed remnants of suprarenal cortex were seen.

The tumour tissue was cellular, with large areas of haemorrhage but with little necrosis. The cells were arranged in solid masses (Fig. 2), often broken up by sinusoids or areas of haemorrhage: in a few areas, whorls and columns of cells were found. In still other areas, the cellular pattern of the suprarenal cortex was simulated (Fig. 3). The cells were of various types: the majority (Fig. 2) were large, polygonal, or round with a slightly basophilic, finely vacuolated cytoplasm and large round or oval, vesicular nuclei: among these cells, there were sinusoids with no reticulin lining. In many parts of the tumour these cells were not pigmented: in heavily pigmented areas (see Fig. 13) some of the tumour cells contained fine refractile granules of light to dark brown pigment, though most of the pigment was present in macrophages lying in the sinusoids, in the interstitial tissue or in fibrous trabeculae and showed as large granules of a light yellowish brown. Other cells were smaller, more eosinophilic and non-vacuolated, with slightly smaller, oval, vesicular nuclei: these cells occurred in strands (Fig. 4) throughout the tumour, mostly round small blood vessels and capillaries. A third type of cell found only very occasionally was suggestive of a ganglion cell (Fig. 5): no other nervous elements were found in the tumour.

Small or large angular cells with much more basophilic cytoplasm and solid oval nuclei were occasionally observed, compressed between the larger cells. With chrome fixation and Sevki's modification of Giemsa's stain, the cytoplasm of these angular cells stained diffusely green. With a similar technique, in some of the main type of cells, the small green granules typical of chromaffin

FIG. 1.—Phaeochromocytoma (Case 1), cut surface. Smooth ovoid tumour with two large cystic spaces: tumour tissue greyish pink with many small haemorrhages. ×½.

FIG. 2.—Phaeochromocytoma: field showing large, polygonal or round cells with vesicular nuclei: masses of cells broken up by sinusoids. Haematoxylin and eosin. ×100.

FIG. 3.—Phaeochromocytoma: field in which the cell pattern simulates the suprarenal cortex. Haematoxylin and eosin. ×120.

FIG. 4.—Phaeochromocytoma: strands of smaller, more eosinophilic cells round small blood vessels. Haematoxylin and eosin. ×60.

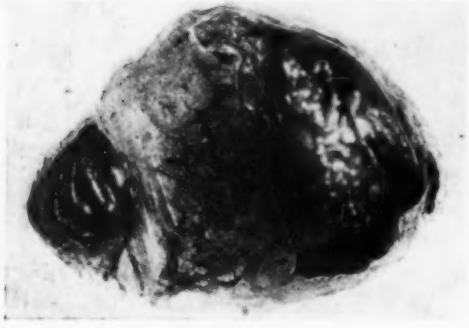

FIG. 1

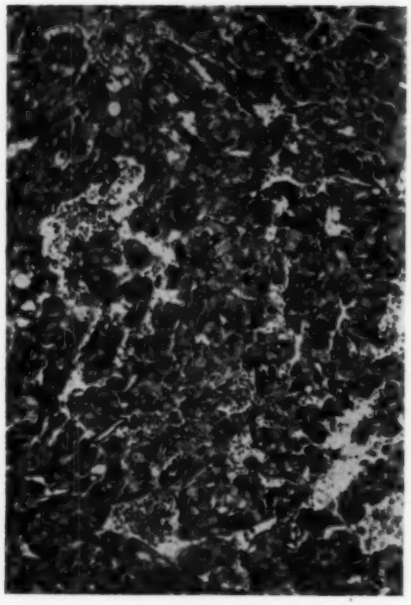

FIG. 2

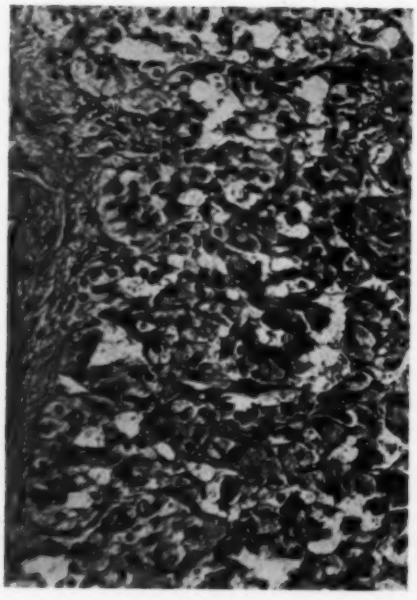

FIG. 3

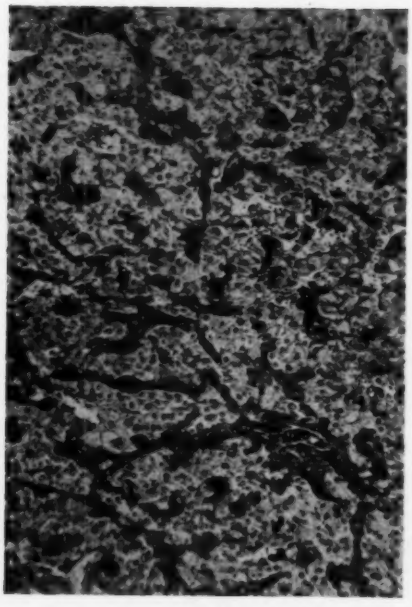

FIG. 4

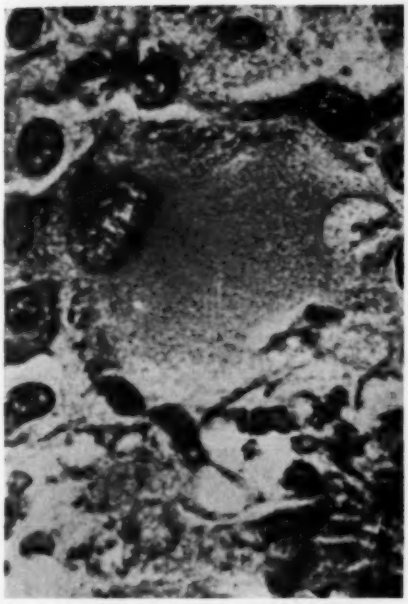

FIG. 5.—Phaeochromocytoma: ganglion-type cell. Haematoxylin and eosin. ×600.

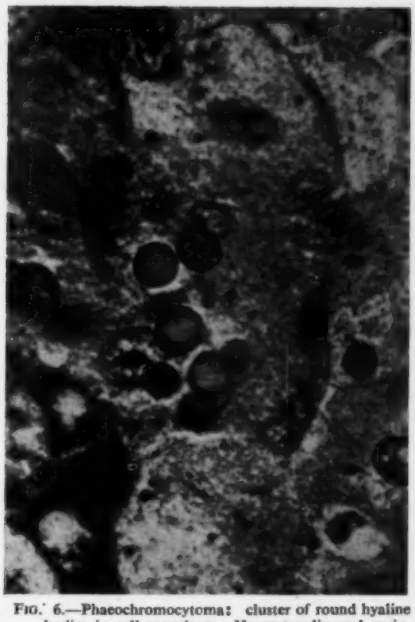

FIG. 6.—Phaeochromocytoma: cluster of round hyaline bodies in cell cytoplasm. Haematoxylin and eosin. ×600.

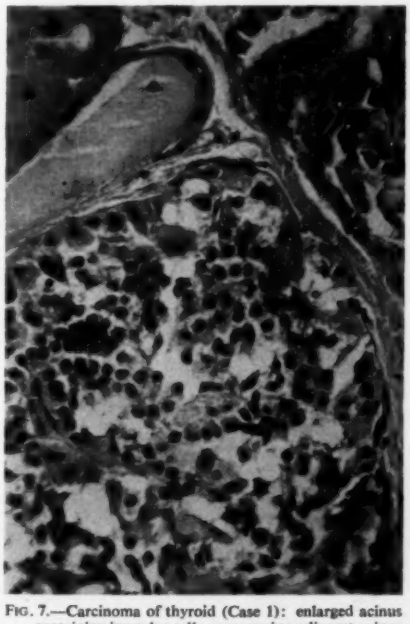

FIG. 7.—Carcinoma of thyroid (Case 1): enlarged acinus containing irregular cells compressing adjacent acinus with normal cells. Haematoxylin and eosin. ×300.

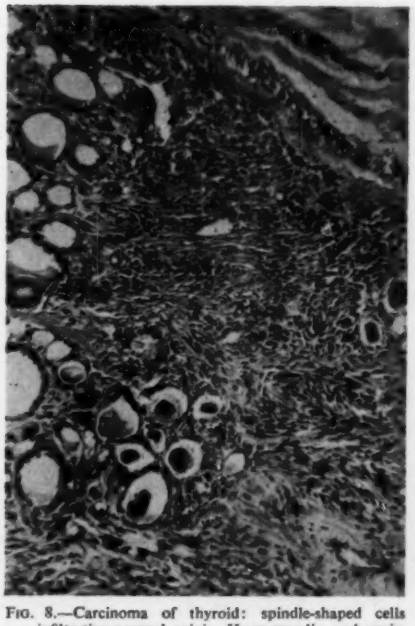

FIG. 8.—Carcinoma of thyroid: spindle-shaped cells infiltrating normal acini. Haematoxylin and eosin. ×100.

were seen and the fluid in some of the sinusoids also gave a green colour. Other granules remained yellowish-brown similar to those in macrophages in the capsule and sinusoids. Also in the cytoplasm of some of the main cells, there were round, hyaline bodies of varying sizes (Fig. 6): they were strongly eosinophilic, stained well with fuchsin and phloxin, and occurred singly or in clusters in individual cells or in groups of cells.

From the above histological characteristics, the typical clinical history and biological assay, this tumour was a true phaeochromocytoma of the suprarenal medulla.

The cervical tumour was a somewhat flattened pinkish-grey mass, $6 \times 4 \times 3$ cm.: on section, the outer third consisted of firm grey tissue with colloid material, while the centre was occupied by irregularly whorled, softer, pinkish-white tissue.

The histological picture was that of carcinoma developing in a simple adenoma of the thyroid (Figs. 7 and 8).

Second Case Report

F. P., a man aged 62 years, presented with a slowly growing mass of two years' duration in the right groin: it was painless and no other lymphadenopathy or abnormal physical sign could be detected. At operation (July 9, 1952), it appeared to be a melanoma. Full clinical investigation at that time, however, failed to disclose a primary tumour in skin, mucocutaneous junctions, or eyes. In view of the superficial histological similarities to the tumour in the first case, the possibility of a phaeochromocytoma was raised, but clinical investigation gave no evidence of such. The patient remained perfectly well and at work until August, 1953, when throbbing pain developed in the right side and numerous bluish nodules appeared in the skin and subcutaneous tissues. One such nodule was excised from the left groin on September 19, 1953. Repeated search for a primary melanoma in skin or eye again gave negative results. His general condition now deteriorated rapidly and he died on October 19, 1953.

Necropsy showed a somewhat emaciated elderly male with multiple subcutaneous bluish nodules, most numerous over the shoulders and anterior chest wall: section of the skin showed that, while most were under the true skin, a few were in its deeper layers although none involved the epidermis: they consisted of soft brown and white tumour nodules with areas of necrosis and haemorrhage. None of these nodules had been apparent at the time of his first admission, 18 months before. Operation scars were seen in both groins. Scars from shrapnel wounds from the 1914–18 war were present on both legs, one 10 cm. in length over the right tibia where an operation had been required, the others small (1 cm. in length) on the back of the left leg. No other scars were found and this agreed with his story that he had had no other operations. Both legs were oedematous. The finger- and toe-nails were all removed and the subungual areas examined carefully for a possible primary melanoma, with negative result. The whole skin surface and all mucocutaneous junctions were again examined meticulously without success.

Several small pigmented tumour deposits (2 mm. in diameter) were present in the heart and pericardium, which were otherwise normal. Chronic fibroid phthisis was found in the upper lobes of both lungs and the lower lobes showed small areas of early infarction (due to emboli from thrombosed leg veins); the hilar glands showed neither caseation nor tumour infiltration, but one anterior mediastinal

FIG. 9.—Melanoma of suprarenal (Case 2), posterior cut surface. Large lobulated tumour with grey, red, and brown areas. ×3/5.

gland was enlarged to 2 cm. in diameter with pigmented tumour tissue. Two small pigmented tumour deposits were seen in the right pleura, posteriorly, while adhesions were found over both upper lobes.

The peritoneum contained 500 ml. clear yellow fluid and several small secondary tumour deposits were present. The mesenteric glands showed slight enlargement with pigmented tumour. The stomach and intestines were normal. The liver was enlarged due to terminal congestion, but no metastases were found in it: the gall-bladder and bile ducts were normal. The spleen was soft and congested and no metastases were seen. One nodule (2 cm.) of pigmented tumour was present in the left kidney, none in the right. The bladder had two small (2 mm.) secondary nodules in the submucosa. No secondary tumour was found in the marrow of vertebrae, ribs, or skull.

The right suprarenal was replaced by a large lobulated tumour, $17 \times 12.5 \times 10$ cm., weighing 900 g., with a smooth surface (Fig. 9). On section, the mass was trabeculated and consisted of soft, brownish-black tissue with extensive areas of necrosis and haemorrhage: no cysts or crystals were present. To the naked eye, no suprarenal tissue was obvious. The left suprarenal was slightly enlarged and contained one macroscopic nodule (2 cm.) of pigmented tumour tissue.

In the meninges, one nodule (0.5 cm.) of pigmented tumour was present overlying the left sensory cortex: no other abnormality was found. In the brain, several small nodules (c. 0.4 cm.) were scattered throughout both cerebral hemispheres with one larger deposit (2.5 cm.) at the posterior horn of the left lateral ventricle. The orbits showed no tumour deposits. Both eyeballs were bisected, examined carefully *in situ* and the posterior halves removed for histological examination, all with negative results for any tumour tissue.

Histology of Tumour of Groin.—This, apparently encapsulated, measured $6 \times 5 \times 3$ cm., and was brown, with black areas. On section, white and brown nodules of tumour tissue were present round the periphery with necrosis and haemorrhage in the centre. An attempt at biological assay on a portion of the tumour failed for technical reasons.

The surrounding tissue consisted of compressed and fibrosed lymphoid tissue, infiltrated in parts by nodules of tumour showing great variation in cellular detail. In infiltrating areas (Fig. 10), the tumour tissue was composed of sheets of large

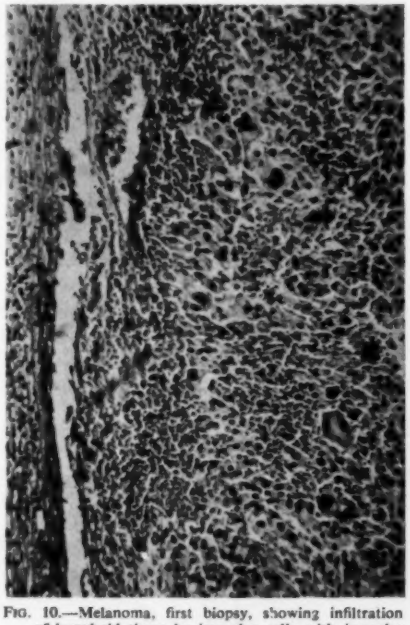

FIG. 10.—Melanoma, first biopsy, showing infiltration of lymphoid tissue by irregular cells with irregular nuclear figures. Haematoxylin and eosin. ×100.

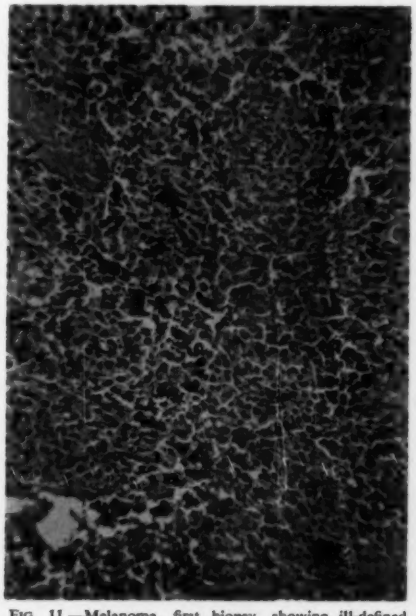

FIG. 11.—Melanoma, first biopsy, showing ill-defined whorls of tumour cells and irregular sinuses. Haematoxylin and eosin. ×100.

irregular polygonal cells with foamy or finely granular cytoplasm and large hyperchromatic oval nuclei in which mitotic figures were fairly numerous: irregular giant cell forms were found. In the necrotic areas, the surviving cells were arranged mostly in whorls in which the central cells were small, irregular, and deeply basophilic, while around them were medium-sized or large, irregular, or polyhedral cells, often in layers like transitional-cell carcinoma: in a few areas, however, there appeared to be an acinar arrangement around sinuses without reticulin lining (Fig. 11) and the cells closely resembled those seen in parts of the phaeochromocytoma. Pigment was present in considerable amount, though with peculiar distribution: it was present in all types of cell described above in some parts and completely absent from similar cells elsewhere: it occurred as fine black dots in the cytoplasm of some cells or as large yellowish-brown granules in others. It was present also in the edges of the dense fibrous trabeculae round the periphery, here mostly as large brown or black granules in compressed macrophages. None of the pigment gave the iron reaction.

In view of the presence of lymphoid tissue, this was a secondary tumour in the inguinal glands. With the knowledge that no primary melanoma could be found clinically, and because of the resemblance of some parts of the tumour to the phaeochromocytoma in the first case, post-chroming and Sevki's stain for adrenaline precursor were tried on the sections and the granules stained bright green. The fallacies of this technique were not realized at that time and it was suggested that the tumour was a secondary phaeochromocytoma or a secondary carcinoma from a primary in the suprarenal.

Subcutaneous Nodule from the Inguinal Region.—This was a greyish-black nodule, the histological appearances of which were similar to those in the previous biopsy. It was realized by this time that post-chroming of tissues allowed non-chromaffin pigment with reducing characteristics, e.g., melanin, to stain green with Sevki's method and it was now accepted that both the excised tumours were secondary malignant melanomata.

Tumour of Right Suprarenal.—The tumour of the right suprarenal showed extensive areas of necrosis and much haemorrhage. Some sections from the periphery showed a broad, dense, fibrous capsule, containing abundant refractile dark brown pigment: in places outside this capsule, small compressed and fibrosed remnants of suprarenal cortex were found. Other peripheral sections showed tumour cells directly infiltrating loose fibrous and fatty tissue. Apparently, this tumour had been growing slowly but had recently assumed more rapidly infiltrating characteristics.

The tumour was composed of masses of cells arranged in sheets and lobules with fairly numerous capillaries and enclosed in bands of dense or loose fibrous tissue. The cells varied greatly in size and shape (Fig. 12), mostly large and polygonal, but without a predominant cell type: the nuclei were large and round or oval, hyperchromatic and with

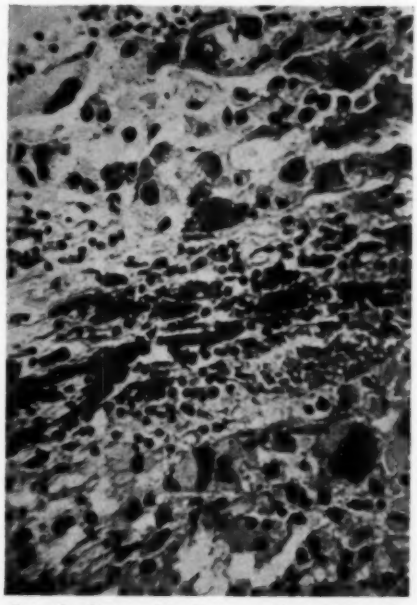

FIG. 12.—Suprarenal melanoma with irregular cells, mostly large and some pigmented; dense pigment is present in a fibrous band across the centre. Haematoxylin and eosin. × 300.

many mitotic figures, some of which were bizarre. In the centres of the lobules and alongside small blood vessels, there were small rounded basophilic cells with small dense nuclei as in the groin biopsy. In many areas, some of the large cells contained fine granules of dark brown pigment, sometimes diffusely dispersed, sometimes only in parts of the cells. In these areas, larger granules of the pigment were concentrated in cells either centrally or at the periphery and in the surrounding fibrous tissue: details of these latter cells were completely obscured by the pigment and they were probably macrophages. In several of the loose fibrous areas, the presence of deeply pigmented cells with no tumour near suggested that some areas of tumour had

undergone necrosis, leaving the pigment in macrophages. In some of the tumour cells, rounded, hyaline, slightly basophilic bodies were seen, but only near necrotic areas, and they showed none of the staining characteristics of the eosinophil hyaline bodies in the phaeochromocytoma of the first case: they appeared to be a degenerative phenomenon.

In the left suprarenal, the mass of tumour seen naked-eye was situated in the medulla, with some fairly dense fibrosis between it and the cortex, but there was also recent infiltration around in some parts. Several small foci of tumour cells were scattered throughout the cortex and medulla. The appearances suggested that the tumour here was secondary to that in the right suprarenal. The histology of all the other deposits throughout the body was similar to that of the mass in the right suprarenal.

It was now believed that the diagnosis was either a primary melanoma of the right suprarenal or, if such a primary is not acceptable (Willis, 1948), a primary pigmented tumour of the suprarenal, e.g., a phaeochromocytoma, in which the physiological activity had been replaced by malignant activity. The final answer appeared to lie in the nature of the pigment, which looked very similar to that in the phaeochromocytoma, and so further investigation of this point was undertaken.

Further Investigation of Pigment Histology

Iron.—The great bulk of the pigment under investigation did not give a positive Prussian blue reaction and so did not contain free iron. Only a few isolated granules in each tumour gave a positive reaction, compatible with haemorrhages into the tumours.

Chromaffin Reaction.—In the suprarenal tumours from both cases the colour was predominantly brown and it was difficult to say if further darkening took place during fixation with chrome salts. On subsequent staining with Sevki's modification of Giemsa's stain, in the first case most of the small and a few of the large granules stained green though the majority of the large granules remained light yellowish-brown: fine green granules were also seen in some cells which showed little or no obvious pigmentation without this technique. This suggested the presence of two types of pigment, one, the chromaffin, occurring mainly as fine granules, and the other (present in much greater proportion), occurring as fine and large granules. In the second case, on the other hand, both small and large pigment granules stained green. Sections of the first biopsy from blocks which had not originally been fixed in a chrome solution, but chromed after formalin fixation, also showed these fine green granules. Blocks from two known melanomata were also chromed after formol-corrosive fixation and sections stained by Sevki's method: the melanin granules stained green. This finding emphasizes that the chromaffin reaction is the original darkening in the chrome fixative and that the later staining by Sevki's method is not specific for granules of chromaffin. This point must be noted in a pigmented tumour in which the diagnosis of phaeochromocytoma is a possibility.

Periodic - Acid - Schiff Technique. — With the periodic-acid-Schiff (P.A.S.) technique, a distinct difference was observed in the pigment of the tumours from the two patients. In the first case, both fine and large granules of pigment gave no reaction, although the cytoplasm of the cells was occasionally positive. Thus neither type of pigment in the phaeochromocytoma was positive with P.A.S.

In the second case, the fine cellular granules remained brown, though sometimes the cell cytoplasm stained pink or red: the great majority of the large granules in cells in the tumour masses and in the stroma were very definitely reddish-brown: this behaviour of the pigment would suggest that it might be melanin, which, according to Pearse (1953), gives a positive P.A.S. reaction only when in melanophages. In sections from the control melanomata, the pigment behaved in a similar fashion.

Bleaching with 10% Hydrogen Peroxide.—The results obtained by using this technique (Pearse, 1953) showed a sharp differentiation in the type of pigment in the two cases. Most of the pigment from the first case was not bleached after 48 hours' treatment, both the large granules and some of the fine particles remaining unaffected (Fig. 13). This resistance to bleaching excluded melanin as being the bulk of the pigment in the phaeochromocytoma and further suggested that it was not of simple chromaffin type. The pigment from the second case, on the other hand, showed practically complete bleaching at 48 hours (Fig. 14), most of the colour coming out between 24 and 48 hours, thus confirming that the pigment was a melanin. This observation was corroborated by simultaneous control tests on pigment from the two known melanomata.

Solubility.—While using Gordon and Sweet's method of staining reticulin for the arrangement of the tumour cells, a clear difference in the reaction of the pigments from the two cases was noticed. In the first case, while most cells showed fine argyrophil granules (presumably chromaffin), large

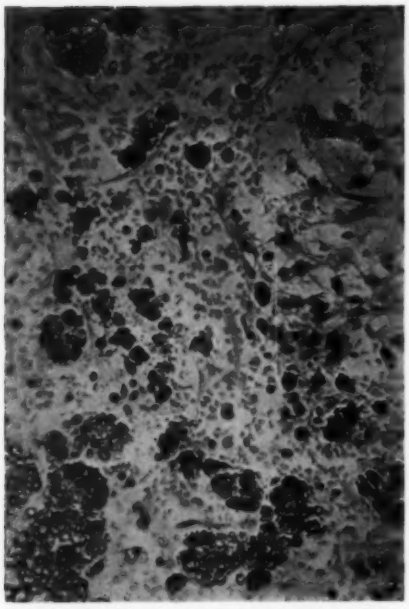

FIG. 13.—Phaeochromocytoma: pigment granules resisted bleaching for 48 hours with 10% hydrogen peroxide. Dilute carmalum stain. ×300.

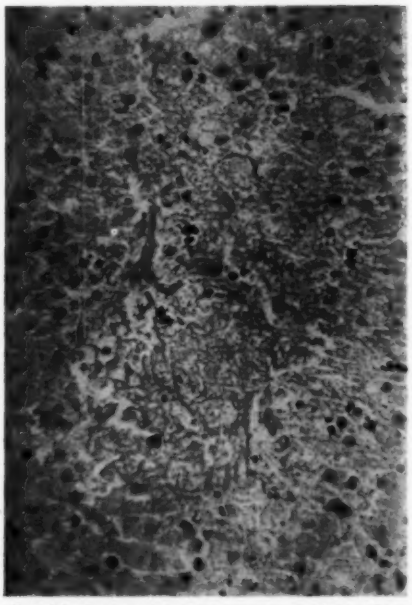

FIG. 14.—Suprarenal melanoma: great majority of pigment granules bleached after 48 hours in 10% hydrogen peroxide (cf. Fig. 12). Dilute carmalum stain. ×300.

granules of pigment were not affected by the procedure, but remained refractile and yellowish-brown.

In the second case, and in the two known control melanomata, however, many of the small granules of pigment stained black, while others, especially the large granules, had disappeared completely. The interpretation of this finding appeared to be that some of the melanin granules showed argyrophilia while others were dissolved by the alkali used in this method. It would appear that when the pigment was present in macrophages it was somehow altered and gave a different reaction when subjected to alkali and to the P.A.S. technique.

Discussion

These two suprarenal tumours were both large, brown, and showed cystic or necrotic spaces. Histologically, compressed cortical tissue was found outside the capsules in both. The tumour cells showed great variety in size and shape and there was much pigment, mostly in macrophages in the capsules and trabeculae. Here the resemblances ended. For, in the first case, the cells were differentiated into several distinct types (Figs. 2, 4, and 5), the nuclei were well formed, and there was no evidence of malignancy. In the second case, the cells showed greater variety without distinct types (Figs. 10, 11, and 12), the nuclei were often irregular, and there was definite evidence of malignancy. Nevertheless, distinction between the two tumours was very difficult, partly on account of the absence of biological assay in the second case, partly because of the close chemical relationship between the pigments in the two cases.

From the clinical history of the first case and the histological and biological findings in the suprarenal tumour, it was a phaeochromocytoma. Only a few points require mention in view of the recent literature on the histology of this tumour (Blacklock, Ferguson, Mack, Shafar, and Symington, 1947; Symington and Goodall, 1953). One was the presence of pigment: this is virtually ignored in most descriptions of the tumour, though Blacklock *et al.* described the tumour in their fourth case as light-brownish in colour and Hyman and Mencher (1943) stated that when large the tumours were red or dark-brown, greyish when small, but no further details of the pigment were given. Here, the nature of the pigment was investigated purely for comparison with that in the other case, and it was found that a pigment apart from chromaffin was present with the following reactions: not sudanophil, not containing free iron, negative to P.A.S., and not bleached by hydrogen peroxide (10%) in 48 hours.

Thus it may be a type of lipo-pigment, perhaps similar to the granules in a ganglion cell (Pearse, 1953).

Another point of interest was the presence of hyaline bodies, intensely eosinophil, occurring singly or in clusters, in the cytoplasm of the cells. They stained strongly with acid fuchsin, phloxin, and eosin, were positive to P.A.S., not fatty, while with Sevki's stain the small ones were pink and the large ones green or blue, they took a counterstain with Gordon and Sweet's method for reticulin and were still present after 48 hours' bleaching with 10% H_2O_2. They did not appear in any of Symington and Goodall's specimens, but were present in the tumour described by Beer, King, and Prinzmetal (1937), and Ewing (1928) states that they "are usually present" in phaeochromocytomata. They appeared to be similar to bodies found in some cells of the normal suprarenal cortex (Selye and Stone, 1950), though not compatible in morphology or in staining reactions with ingested red blood corpuscles, as these authors had suggested.

The final point about the first case was the persistence of symptoms after removal of the suprarenal tumour and the subsequent development of an adenocarcinoma of the thyroid. The association of these two tumours was first pointed out by Eisenberg and Wallerstein (1932), who reported one such combination in a review of 53 cases of phaeochromocytoma, and later by Symington and Goodall (1953), who found two others (Muntz, Ritchey, and Gatch, 1947, and Rothermich, 1952) in 283 cases of the same tumour, while Beer *et al.* (1937) reported a fourth, but in these the adenocarcinoma of thyroid presented first. The association appears to be more frequent than would occur by chance. The behaviour of the thyroid in patients with phaeochromocytoma is discussed fully by Slessor (1955).

The second case provided a much more difficult problem. At the time of the first biopsy, in view of the complete absence of any other clinical findings and with the histological resemblances to the tumour from the first case, especially the wrongly interpreted reaction to Sevki's staining method after chrome fixation, it appeared logical to suggest the possibility of a malignant phaeochromocytoma. This in itself would have been a rare occurrence; phaeochromocytoma is described as being "usually simple" (Cappell, 1951). Symington and Goodall (1953) accepted reports of only seven malignant examples though none had biological assays of the secondary tumour, while Willis (1948) cast doubt on all cases reported as malignant in view of the lack of biological assays. Robinson and Baker-Bates (1954) reported a case of carcinoma of the suprarenal cortex which might easily have been mistaken for a malignant phaeochromocytoma, especially in view of the fact that a simple phaeochromocytoma may simulate the suprarenal cortex in parts (cf. our Fig. 3 and Robinson and Baker-Bates' Fig. 476A). Biological assay in their case, however, was negative and the histology did not show sufficient pleomorphism of the cells. In the present case, the clinical absence of any signs of hyperadrenalism made it extremely unlikely that such a specialized tumour could lose all its powers of adrenaline secretion in return for the property of malignant growth. The subsequent behaviour of the growth became typical of a melanotic tumour and this was confirmed by the histological features in conjunction with the reactions of the pigment.

This then raised the question of its being a primary melanoma of the suprarenal medulla. Willis (1948) cannot accept this as a primary site for melanoma unless strict requirements are satisfied. In this case, the skin and eyes had been searched repeatedly during life by competent clinicians on many occasions, and again at necropsy the examination was repeated with meticulous care: every nail was removed, the eyeballs opened, the pia-arachnoid examined, all without finding a possible primary growth in the other accepted sites. Muir's *Text-book of Pathology* (Cappell, 1951) gives the suprarenal as a site for primary melanoma, and Ewing (1928) quotes cases reported as melanosarcoma by Davidsohn, Goldzicher, Tuczek, and Maclachlan. From Maclachlan's (1915) description of his own case and his review of the earlier three, there is no doubt that similar precautions as to possible sources of a primary elsewhere were fully taken and these four closely resemble our own case. Smith (1927) has also described a similar growth, and, though he did not examine the eye-balls, the orbits were clear of tumour, and there is little doubt that his case was also primarily in the suprarenal. While there was considerable argument and disagreement as to the exact nature of the earlier tumours, the development of the medulla of the suprarenal from neuroectoderm provides a complete analogy for tumours from this site with skin melanomata, on the basis of Nicholson's (1922 and 1923) and Dawson's (1925) work as accepted by Willis (1948) and Cappell (1951).

Accordingly, we submit that this tumour was a primary melanoma of the suprarenal medulla.

Summary

Two patients had primary pigmented tumours in the suprarenal medulla: the histological character-

istics with special reference to the pigment are described and compared.

In one patient, the tumour was a phaeochromocytoma—simple, with pleomorphism but distinct cell types: in addition to chromaffin, there was another pigment probably of the lipo-pigment group. The tumour was removed successfully. Eighteen months later, a carcinoma developed in a simple thyroid adenoma and was also removed with no recurrence to date.

In the other patient, two biopsies were performed, one from each groin, two years and six months before a primary melanoma of the suprarenal medulla was found at necropsy.

Some difficulties in the differentiation of pigmented suprarenal tumours are pointed out.

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RETICULUM-CELLED MEDULLARY RETICULOSIS IN A YOUNG ADULT

BY

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The clinical picture and histology of the non-lipoid reticulo-endotheliosis of children usually known as Letterer-Siwe disease is well established, but there is dispute as to the origin and nature of the abnormal hyperplastic cells, whether histiocytes or reticulum cells and whether primarily of sinus or medullary origin. These cytogenetic problems have been exhaustively discussed in many publications, notably those of Abt and Denenholz (1936), Robb-Smith (1938), Wallgren (1940), Lichtenstein and Jaffe (1940), Mallory (1942), Jaffe and Lichtenstein (1944), Schafer (1949), and McLetchie (1952). The discussions include much speculation on the connexion between Letterer-Siwe disease, Hand-Schüller-Christian disease, and eosinophil granuloma of bone, but there is little reference to the occurrence of the histological picture of Letterer-Siwe disease in adults. Robb-Smith (1938, 1947) refers to reticulum-celled medullary reticulosis in adults as somewhat resembling typhoid fever in clinical course, with splenomegaly, generalized lymphadenopathy, and death usually within a year, while Scott (1951) describes the condition as more chronic than in children, with massive splenomegaly and lymph node enlargement, anaemia, thrombocytopenia, and leucopenia. The latter author mentions a patient relieved for some 18 months by splenectomy and irradiation. Apart from these brief references the literature contains no detailed case reports of the course of this disease in adults, and it is the object of this communication to present such a case history together with an account of the haematological and cytological findings during life and the information obtained from post-mortem examination.

Case Report

Clinical Course and Investigations during Life.—The patient was a young man aged 19, a farm worker, whose first complaint was of enlarged axillary glands, noticed in January, 1953. There was no relevant past or family history, no pyrexia or constitutional disturbance, and no further physical signs at this stage other than slight splenomegaly. A chest radiograph showed no hilar enlargement. There was no anaemia, the leucocyte count was 6,200/c.mm. with normal differential, the sedimentation rate was normal, and the Paul-Bunnell test negative. Histological examination of a lymph node biopsy showed chronic inflammatory changes of a non-specific nature. The condition remained little changed until April, 1953, when increasing lassitude and cough with sputum developed. The spleen was enlarged to the umbilicus and the liver to three fingerbreadths below the right costal margin. Mantoux tests and frequent sputum examinations for tubercle bacilli proved negative, and a chest radiograph showed no pulmonary infiltration although there was some prominence of hilar shadows. Blood examination revealed mild anaemia (Hb 10 g.%) and a general leucopenia (W.B.C. 1,000/c.mm.); no abnormal or primitive cells were seen. Aspiration of sternal marrow showed high cellularity with hyperplasia of both normoblastic and granulocytic cells, increased mitotic activity, and a suggestion of a granulocyte maturation defect (Figs. 1 and 2). A second attempted lymph node biopsy showed chiefly fatty connective tissue with some inflammatory reaction. In view of the pyrexia, which now ranged up to 103° F., a blood culture and Widal reaction were carried out; results were negative. Over the next month improvement occurred, the spleen and lymph glands became smaller and cough and dyspnoea diminished. A third axillary gland biopsy was performed in May and once again only chronic inflammatory changes were found; Ziehl-Neelsen staining showed no tubercle bacilli. A somewhat fluctuating course was followed until July, 1953, when an attack of varicella supervened. By the end of this infection the clinical state was very poor, with pyrexia of 104° F., dyspnoea, cyanosis, and gross splenomegaly and hepatomegaly, but a further improvement took place in August, and by the end of this month the spleen and liver could scarcely be felt and the lymph nodes were much smaller. At this time an eosinophilia developed, rising to a maximum level of 1,000 eosinophils per c.mm. in the blood, and persisted for about three weeks. No further diagnostic information accrued and

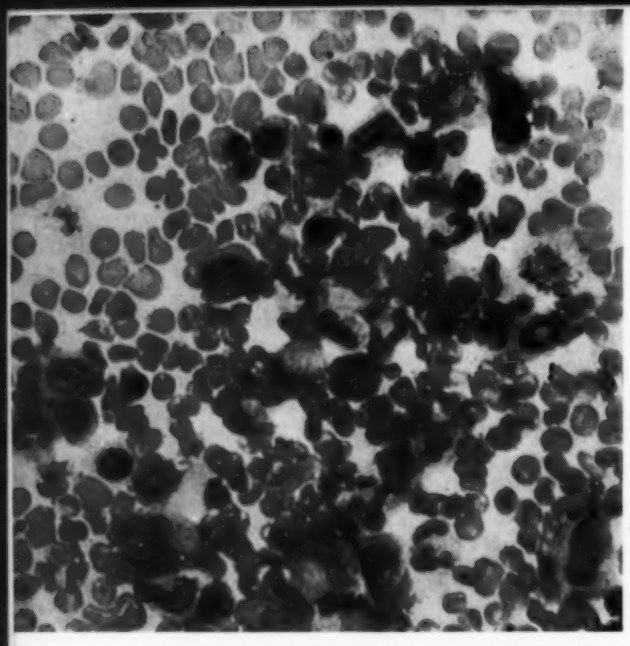
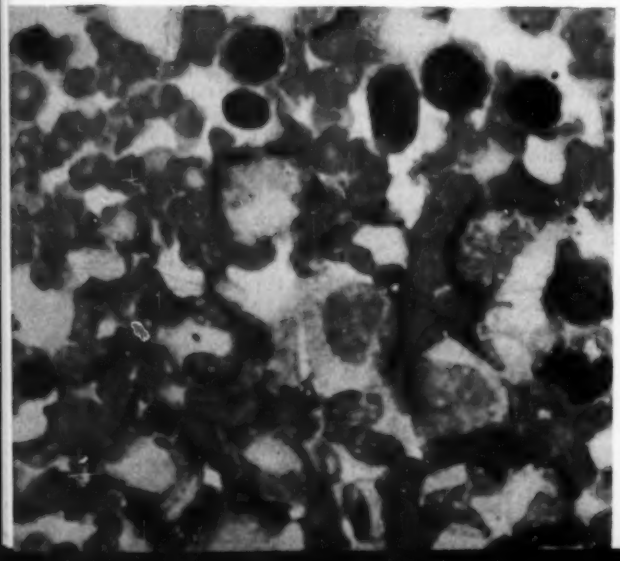


FIG. 1.—Sternal marrow smear showing degree of cellularity in body of film. Leishman, × 280.

the clinical improvement was substantially maintained until November, when a high pyrexia with considerable hepatosplenomegaly and lymphadenopathy recurred. Blood examinations during the next month showed a steady deterioration, Hb falling gradually from 10.5 g.% to 7 g.%, leucocytes from 1,200 per c.mm. to 400 per c.mm., and platelets varying between 100,000 and 50,000 per c.mm. During this period an aspiration biopsy of the spleen was performed, but apart from some increase in macrophages no diagnostic information was obtained. A further bone marrow puncture revealed the persistence of active hyperplasia, with no abnormal cells. The clinical state continued to worsen and the patient died in December, almost a year after the onset of the disease. No firm diagnosis had been made during life, and therapy had initially been directed against a possible tuberculous state. Some encouragement in the use of streptomycin and isoniazid, and, later in the course of the disease, of cortisone, was given by improvements coincidental with treatment, but it seems likely in retrospect that fluctuations in the natural history of the disease were responsible for these misleading impressions, since no drug proved persistently or repeatedly effective.

FIG. 2.—A group of marrow cells of granulocytic and normoblastic series showing normal cytology. Leishman, × 500.



Post-mortem Examination

Apart from general pallor of most organs and the presence of scattered bronchopneumonic lesions, the major abnormalities were confined to the lymphatic glands, spleen, and liver.

There was widespread lymphadenopathy, particularly in the abdomen, where paraaortic glands and groups at the hilum of the spleen and at the porta hepatis were prominently enlarged. Cervical, axillary, and inguinal glands showed moderate enlargement. On section the glands varied from white to greyish pink; they were moderately firm in consistency.

The spleen was grossly enlarged (weight 2,300 g.) and rather soft; some follicular structure could be made out on the cut surface, but there was no macroscopically visible infiltrate.

The liver weighed 2,700 g. and was paler than normal; there was a suggestion of increased pallor at the periphery of the lobules.

The bone marrow of the sternum was normal in appearance and there was some extension of pinkish marrow into the upper part of the femoral shafts.

The cardiovascular, gastro-intestinal, urogenital, endocrine, and nervous systems showed no gross abnormality.

Histology of Post-mortem Material

The lymphatic glands showed histological changes of a uniform character but greatly varying in degree, the changes being least marked in cervical and axillary nodes and most extensive in the abdominal glands. The general architecture of the glands was preserved and recognizable in even the most grossly affected nodes. Many of the follicles contained conspicuous germ centres. The

FIG. 3.—Lymph node section showing medullary invasion by large reticulum cells. Haematoxylin and eosin, × 140.

FIG. 4.—Detailed cytology of reticulum cells from lymph node, showing irregular cytoplasmic outlines and tendency to clumping and syncytial formation. Haematoxylin and eosin, × 500.

FIG. 5.—Bi- and tri-nucleated reticulum cells and occasional cytoplasmic vacuolation in lymph node section. Haematoxylin and eosin, × 500.

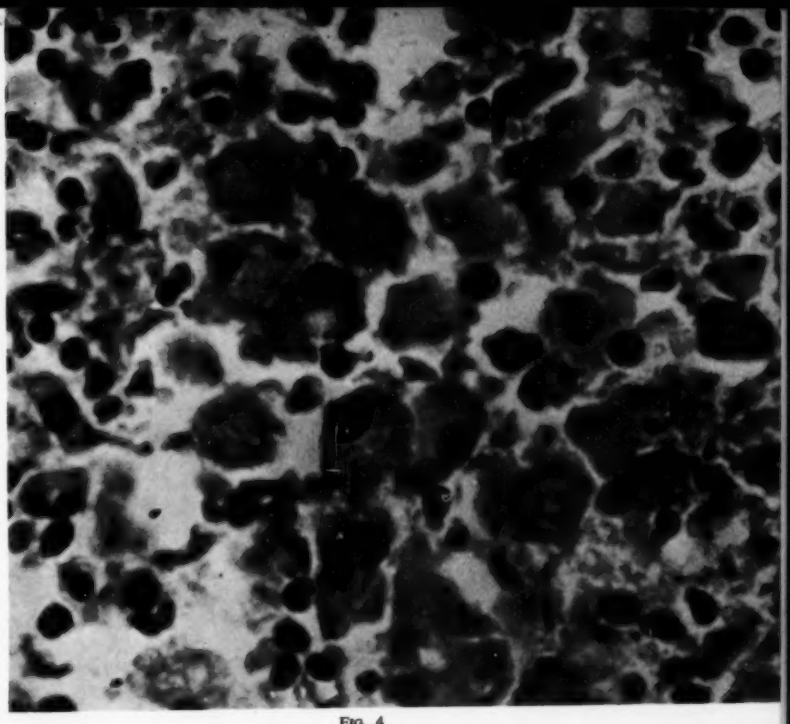


FIG. 4

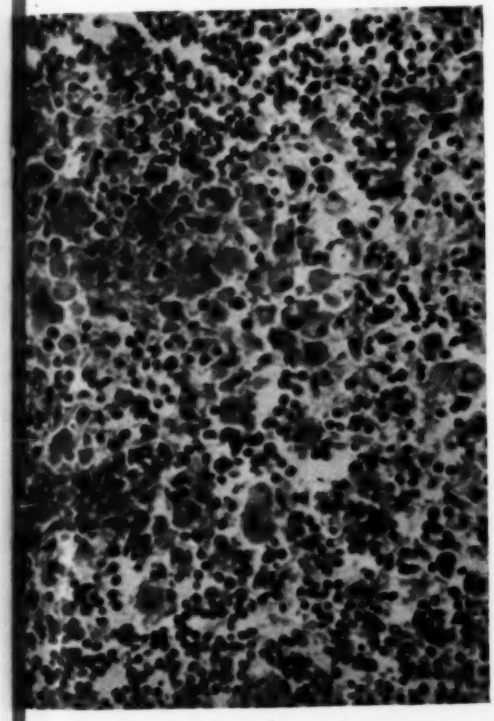
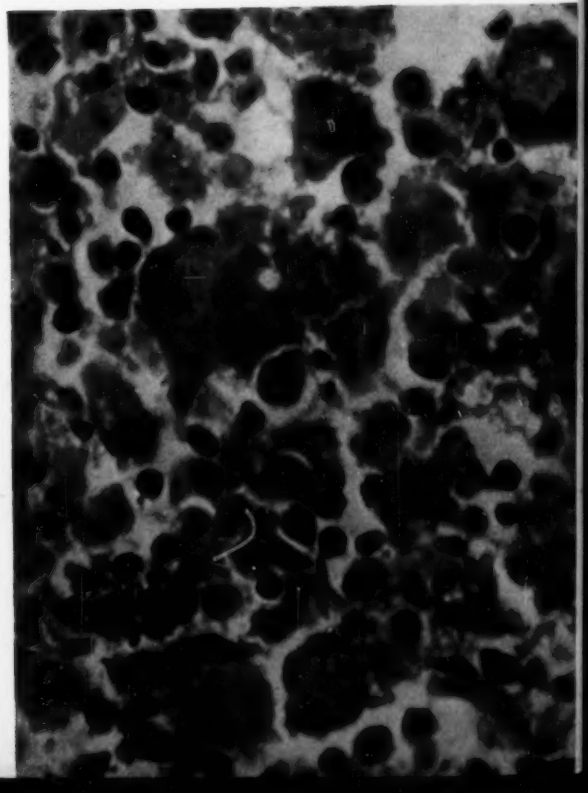


FIG. 3

FIG. 5



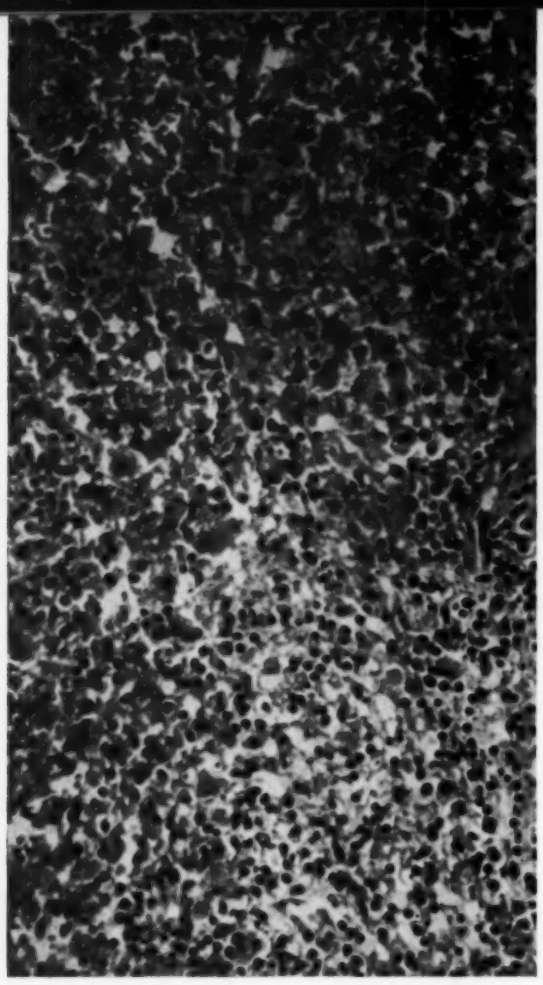


FIG. 6.—General view of reticulum cell hyperplasia in spleen. Haematoxylin and eosin, × 140.

main abnormality involved the medullary tissue, where great numbers of large reticulum cells with abundant eosinophilic cytoplasm and often ragged outlines were found compressing the nodes and sinuses. There were some scattered areas of haemorrhage and occasional erythrophagocytosis, but such phagocytosis was infrequent even where considerable extravasation of red cells had taken place. Some of the proliferating reticulum cells were binucleated and cytoplasmic vacuolation was occasionally seen. In some areas there was a tendency to syncytial form, but multinucleated cells of the characteristic Sternberg-Reed type were not present. The lymph node cytology is illustrated in Figs. 3 to 5.

The spleen showed substantially normal architecture, but contained many collections of large cells of similar appearance to those found in the lymph nodes (Fig. 6).

The liver sections showed some fatty change and moderate infiltration with reticulum cells and lymphocytes, particularly in the region of the portal tracts (Figs. 7 and 8).

The bone marrow appearances were within normal limits and no reticulum cell infiltration could be detected (Fig. 9).

Sections of other organs showed no significant relevant abnormality. Bronchopneumonic changes in the lungs and some focal lymphocytic infiltration in the left kidney were noted.

Discussion

The histological features of this case are those of reticulum-celled medullary reticulosis. There is no lipoid storage and none of the specific clinical or pathological characteristics of Hand-Schüller-Christian disease. Although a transient eosinophilia

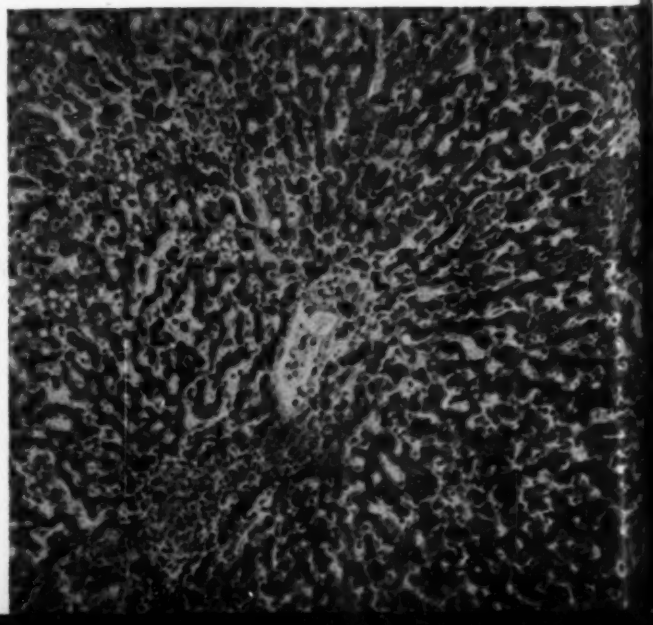


FIG. 7.—Focal areas of infiltration in liver. Haematoxylin and eosin, × 50.

developed at one stage in the disease there is no evidence of eosinophil granulomatosis. The clinical picture and length of course are unlike those of histiocytic medullary reticulosis, and there is no evidence of the very active histiocytic erythrophagocytosis found histologically in this disease. The normal bone marrow findings exclude the group of disorders described by Israëls (1951) under the name of myeloid reticulosis and the many varieties of aleukaemic leukaemia. Such cases as those of Evans, Cipriano, and Ferrell (1949) and Keech and Scott (1953) illustrate the points of resemblance between stem-cell leukaemias and aleukaemic reticulo-endothelioses and many case reports in the earlier literature fall into such intermediate categories. Goldzieher and Hornick (1931) and Dameshek (1933) reviewed over 20 such cases, but Schafer (1949) could find no convincing evidence in previous reports that the reticulo-endotheliosis of adults was the same disorder as infantile non-lipid reticulo-endotheliosis. It is clear that innumerable transitional forms occur intermediately between the classical diseases of the haemopoietic and reticulo-endothelial systems. This is to be expected in systems containing many undifferentiated cells of multiple developmental potentialities, and it is therefore the more interesting to note in the present case the absence of confusing involvement of many cell types and the relative simplicity of the cytological picture.

Summary

A young man of 19 years succumbed to an illness characterized by lymph node enlargement, splenomegaly, hepatomegaly, pyrexia, moderate anaemia, leucopenia, and thrombocytopenia. The bone marrow cytology was within normal limits, lymph node biopsies and splenic puncture failed to establish the diagnosis, and the disease pursued a fluctuating course to its termination one year after the onset. Histological examination of post-mortem material showed reticulum-celled medullary reticulosis.

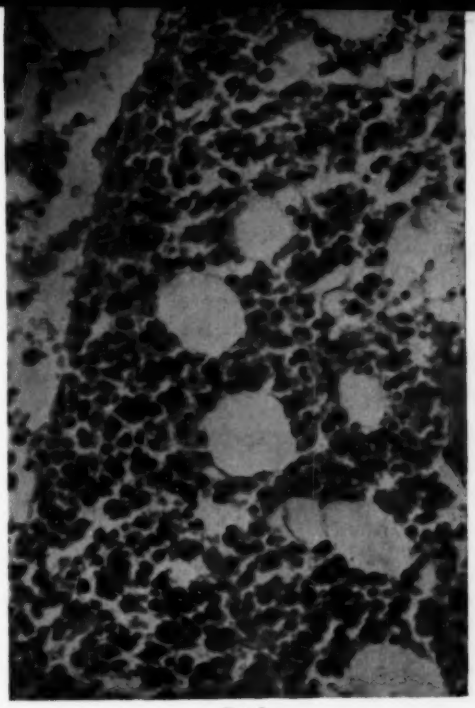


FIG. 9

I wish to record my indebtedness to Dr. Arden Jones, who referred this patient; to Dr. A. M. Barrett and his staff, who performed the post-mortem examination and prepared the sections; and to Sir Lionel Whitby for his constant interest and advice.

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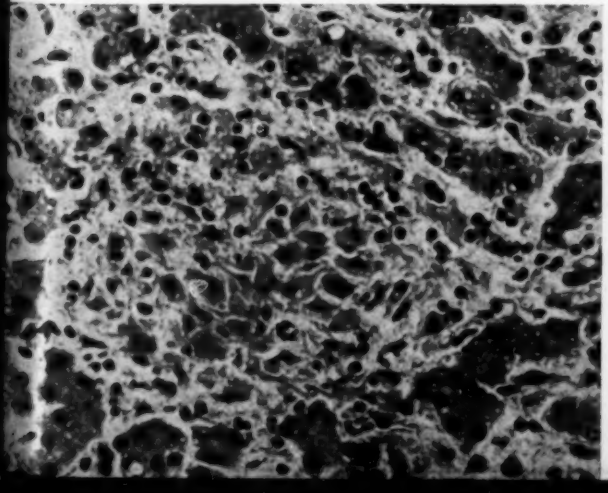

FIG. 8.—Cytology of a focus of reticulum cell and lymphocyte infiltration in liver. Haematoxylin and eosin, × 186.

FIG. 9.—Section of rib showing normal marrow cellularity. Haematoxylin and eosin, × 280.

FIG. 3

J. clin. Path. (1955), **8**, 104.

NASOPHARYNGEAL PLASMACYTOMA WITH EXTENSIVE SKIN METASTASES

BY

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(RECEIVED FOR PUBLICATION AUGUST 5, 1954)

Myelomatosis is not an uncommon condition, but widespread extraskeletal foci are unusual and cutaneous deposits are rare. In 1936 Aragona reported a tumour composed of plasma cells in the skin of the neck of a 6-year-old girl, with no recurrence after five years. Apart from this possible example, primary cutaneous plasmacytoma has not been described. Secondary cutaneous deposits in myelomatosis have been recorded only 16 times (Table I). Two further examples are presented here.

Case Reports

Case 1.—Early in 1943 a man aged 48 years developed pain over the left antrum and intermittent left nasal obstruction with a purulent discharge. Examination showed a polypoidal mass in the left posterior choana and opacity of the left antrum to transillumination; a left-sided ethmoidectomy and resection of turbinates was performed.

In July, 1944, the patient had a profuse left-sided epistaxis and a vascular tumour was found in the left side of the nose and left antrum. Biopsy suggested a lymphoid neoplasm. The chest was radiologically normal and radiotherapy was given to the nasal region with considerable clinical improvement.

In May, 1945, expanding osteolytic lesions of the tenth left rib and the seventh thoracic vertebra were found and irradiated.

The nasal tumour recurred in March, 1947, and was now confirmed as a plasmacytoma. Despite further irradiation both sides of the nose became completely obstructed (October, 1947). A radium pack was applied to the nose (1,000 mg. hours).

In January, 1948, the patient complained of pain in both legs and general fatigue. The nasal airway was patent, but a smooth mass was felt in each tibia and on the vertex of the skull. Radiography showed osteolytic lesions in these regions.

Laboratory investigations showed: haemoglobin 12.7 g. per 100 ml., red blood cells 4.64 m. per c.mm., white blood cells 6,650/c.mm. (differential count normal); plasma proteins 8.25 g. per 100 ml. (albumin 4.60, globulin 3.65); no Bence-Jones proteinuria.

Shortly after irradiation of the affected bony sites the patient sustained a pathological fracture of the head of the right tibia, and *x*-ray examination showed, in addition, destruction of the distal end of the right radius and of some carpal bones. A further course of radiotherapy was given.

He was readmitted in January, 1949, having lost 1 stone (6.38 kg.) in a year, and complaining of pain in the right arm, left leg, and head, and a constant serosanguineous nasal discharge. Scattered in the skin of the trunk were tender, bluish-purple, or dusky-pink sessile nodules, measuring 1–5 cm. in diameter. A course of intravenous nitrogen mustard hydrochloride (di(2-chloroethyl)methylamine hydrochloride) was administered (28.8 mg.) with general subjective improvement, cessation of the nasal discharge, and diminution in the size of a left tibial mass.

The nasal discharge returned in May, 1949, with pain, swelling, and limitation of movement of many joints, and multiple skin tumours on trunk and limbs (Fig. 1). Widespread bony destruction was seen radiologically, especially about the ends of the long bones, and there was a pathological fracture of the upper end of the right radius (Fig. 2).

Investigation now showed: haemoglobin 12.7 g. per 100 ml., white blood cells 6,500/c.mm. (differential count normal); serum calcium 10.4 mg. per 100 ml. (5.2 mEq. per l.), serum phosphorus 2.72 mg. per 100 ml. (1.6 mEq. per l.), alkaline phosphatase 3.6 K.-A. units; plasma proteins 8.25 g. per 100 ml. (albumin 4.0, globulin 4.25); E.S.R. (corrected for Hb) 25 mm. in the first hour (Wintrobe); no Bence-Jones proteinuria. Repeated marrow punctures (sternal and vertebral) did not yield cellular marrow. A skin tumour was confirmed histologically as a plasmacytoma.

After a second course of intravenous nitrogen mustard (25.6 mg.) the skin nodules regressed and the fracture united. Two courses of urethane (total 164 g.) were then given orally. However, by September, 1949, the skin nodules were again larger and more widespread, and there was extensive destruction of the lower end of the left femur and the upper end of the left tibia (Fig. 3). The E.S.R. was 55 mm. in the first hour (Wintrobe), and the total and differential white blood counts were normal, but the urine now contained

FIG. 1.—Case 1: myelomatous deposits in skin of lower chest and upper abdomen.

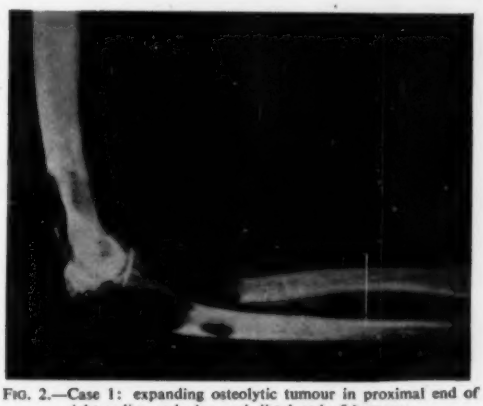

FIG. 2.—Case 1: expanding osteolytic tumour in proximal end of right radius and ulna and distal end of humerus.

Bence-Jones protein and the plasma proteins had risen to 10 g. per 100 ml. (albumin 4.4, globulin 5.6). A sternal marrow aspirate showed normal haemopoiesis with no increase of plasma cells.

Despite the administration of urethane (69 g.), "R.48" (β-naphthyl-bis (2 chloroethyl)-amine (9.7 g.), pentamidine isethionate (2.0 g.) and further "R.48" (10.5 g.), the patient's condition steadily deteriorated. The nasal discharge returned, the skin nodules ulcerated, further bony lesions developed, and he died on February 8, 1950.

Necropsy

Numerous skin tumours, up to 6.0 cm. diameter, were present over the trunk and lower limbs. They were raised plaques or sessile nodules, sometimes ulcerated and frequently purplish. Throughout the skeleton there were widespread, often expanding, tumours, particularly at the ends of the long bones, but the only gross extraskeletal growths, apart from those in the skin, were a small nodule above the upper pole of the right kidney and another in the pancreas.

Histology

All tumours were composed of closely packed cells in a fine reticular stroma. These rounded cells (Fig. 4) resembled primitive plasma cells, with slightly basophilic cytoplasm, sometimes with a perinuclear halo; the large eccentric nuclei contained irregularly condensed chromatin and often a distinct nucleolus. A few binucleated cells and

FIG. 3.—Case 1: destruction of the lower end of the left femur and head of the left tibia.

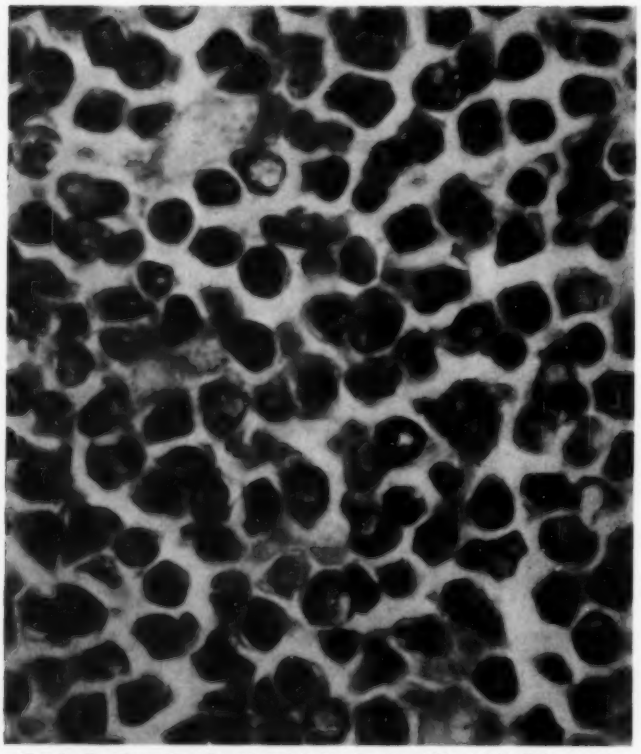

FIG. 4.—Case 1: section of a skin nodule showing the cellular detail of the growth. Haematoxylin and eosin, ×900.

mitotic figures were seen. The skin tumours extended up to the epidermis and stretched it, enveloping and destroying appendages, with local infiltration, and a tendency to form long narrow trabeculae. There was no infiltration of the liver or kidneys.

Case 2.—A 60-year-old labourer first attended hospital in July, 1951. Previously well, he now complained of left nasal obstruction of two weeks' duration, with infraorbital pain, headache, epistaxis, and purulent nasal discharge. Vascular polypoid tissue, seen in the left antrum and ethmoid, was biopsied and found to be a plasmacytoma. There was radiological evidence of bony destruction in the left antrum, in the ethmoidal sinuses, and in the left orbit, but no other skeletal lesion was found. A sternal marrow aspirate showed no evidence of myelomatosis, there was no anaemia, the plasma proteins were normal (albumin 4.3, globulin 2.6 g. per 100 ml.), and Bence-Jones proteinuria was not detected.

Following irradiation of the left orbital region and sinuses there was considerable bony regeneration in these areas. The patient remained well until May, 1953, when he complained of recent anorexia, vomiting, and pain in the right groin, hip, and shoulder. There was ptosis of the left eyelid. Over the skin of the abdomen, chest, back, and thighs there were numerous firm tumours (Fig. 5), one of which was removed and found to be composed of myeloma cells similar to those seen in the original biopsy. A normochromic anaemia (Hb 6.3 g. per 100 ml.) was present, but the total and differential white blood counts were normal and a sternal marrow aspirate showed a poorly cellular marrow with no evidence of myelomatosis. Bence-Jones proteinuria was not found and no osteolytic lesions were seen radiologically in the pelvis or spine.

The left orbital region and the skin tumours were irradiated, but the patient died (May, 1953) after bleeding during the night from a large ulcerated mass in the left thigh.

Necropsy

Numerous firm polypoid or sessile tumours, 0.5–4.0 cm. in diameter, were present in the skin of the abdomen, chest, back, axillae, and upper thighs. They were mainly white and cellular, but others were ulcerated and necrotic, with purplish discoloration from haemorrhage. A huge, white, retroperitoneal mass, partly necrotic and haemorrhagic, completely surrounded both kidneys and replaced the perinephric fat. Gross left hydronephrosis had been produced by extension of the growth into the peripelvic fat and subsequent ureteric obstruction. Many of the lymph nodes about the splenic hilum, coeliac axis, stomach, pancreas, and aorta, and in the mediastinum, axillae and inguinal regions were replaced by neoplasm, and very numerous tumours, up to 1 cm. in diameter, were scattered in the omentum and mesentery. Single tumours were present both in the pyloric submucosa of the stomach and below the pericardium of the left ventricle. The lower lobe of the right lung was collapsed and fibrosed due to unresolved pneumonia.

The sole skeletal lesion was a pathological fracture and fusiform tumour of the right eighth rib. Red marrow filled the ribs and vertebrae, but the right femoral cavity contained gelatinous fatty marrow with mere traces of haemopoietic tissue and no evidence of myelomatosis.

FIG. 5.—Case 2: the appearance and distribution of the cutaneous tumours.

Histology

The tumours were composed of closely packed, rounded or ovoid cells with amphophilic cytoplasm and often a perinuclear halo (Fig. 6). The eccentric nuclei usually contained dense chromatin strands and often an eosinophilic nucleolus. The cells showed some variation in size and shape, with occasional multinucleated and mitotic forms, and generally resembled primitive plasma cells, the cytoplasm staining positively with the pyronin of Unna–Pappenheim's stain. The cutaneous tumours lay immediately deep to the dermal layer, extending into it and eventually ulcerating the epidermis. Occasional myeloma cells were seen in the liver sinusoids and splenic pulp, but not in the kidneys. Marrow from the sternum, vertebrae, and right femur was hypoplastic and gelatinous, with few mature polymorphs, and no evidence of myelomatosis.

Discussion

Our two cases show a striking similarity, although the clinical features differ in several important respects from both classical multiple myeloma and solitary plasmacytoma. In both cases the initial lesion was a soft-tissue plasmacytoma of the maxillary or ethmoidal region, followed later by the appearance of widespread cutaneous plasmacytomata and involvement, to a differing extent, of bone and lymph nodes. The marrow in both cases was poorly cellular and aspirates during life were not diagnostic. The plasma protein fractions remained normal in one case, and in the other showed the usual alterations only late in the illness, despite widespread lesions.

Extramedullary plasmacytomata are uncommon tumours, affecting males more frequently than females, and generally occurring in the mucous membranes of the upper air passages. Their histological appearance gives little clue to their future course, which varies from complete recovery

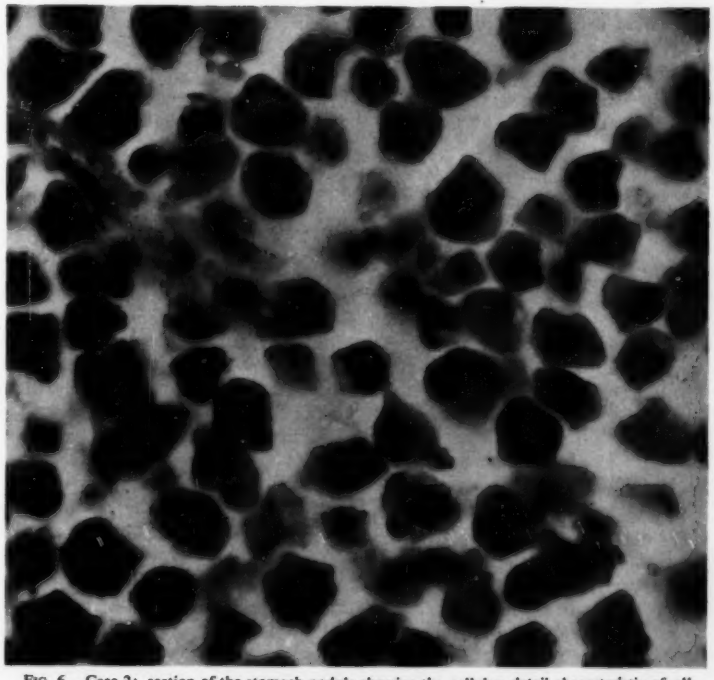

FIG. 6.—Case 2: section of the stomach nodule showing the cellular detail characteristic of all the lesions. Haematoxylin and eosin, ×1,400.

TABLE I

CASES REPORTED IN THE LITERATURE SHOWING CUTANEOUS MYELOMA DEPOSITS

Author	Age	Sex	Skin Lesions	Bence-Jones Protein	General Condition
Renzi (1911)	—	—	Nodules in abdominal wall	Absent	Myelomatosis of bones and lymphoid tissue (P.M.)
Kreibich (1914) ..	78	F	16 tumours in skin of the limbs; infiltration of skin of nose, upper lips, and left lower eyelid	—	Myelomatosis of bones, lymph nodes and meninges (P.M.)
Nicholls (1927)	74	F	Small nodules up to 1.25 cm. diam. in skin of thorax, upper abdomen, and scalp	Absent	Myelomatosis of bones, pleura, lung, ileum, lymph nodes, heart, pancreas, adrenals, liver, and spleen (P.M.)
Piney and Riach (1931) ..	56	F	Helmet-like infiltration of scalp; very numerous small tumours in skin of trunk and limbs	—	Plasma cell leukaemia: W.B.C. 42,250/c.mm. 35% plasma cells. Myelomatosis of bone, liver, lung, tonsil, heart, parotid, prostate, and kidney (P.M.)
Mitsufuji (1936)	57	F	Several tumours of skin of scalp and trunk	Absent	Osteolytic skull lesions
Duvoir *et al.* (1938) ..	59	M	Violaceous tumours in skin above left elbow and in neck	—	Myelomatosis of bones
Loubat (1938)	59	F	3 subcutaneous masses in neck, back, and popliteal space	—	Myelomatosis of bones
Ringertz (1938)	57	F	Tumours in skin of ear only		Case 385E. Began as laryngeal plasmacytoma. Myelomatosis of bones and trachea
Kin (1939)	—	—	Large, umbilicated, ulcerated tumours of skin of face and back; resembled mycosis fungoides	—	Myelomatosis
Sasaki (1939)	75	M	Reddened masses developed terminally in skin of trunk and limbs; firm and occasionally ulcerated	—	Myelomatosis of bones, lungs, liver, spleen, kidneys, adrenals, and lymphoid tissue (P.M.)
Churg and Gordon (1950)	—	—	Subcutaneous tumours (site unspecified)	—	Case 20, myelomatosis of liver, spleen, lymphoid tissue, lung, diaphragm, epicardium, and skeletal muscles (P.M.)
Saltzman and Borgström (1950)	50	M	Several small tumours in skin of abdominal wall terminally	—	Began as nasal plasmacytoma; myelomatosis of bones. Alb./glob. ratio inverted
Campbell (1951)	45	M	Tumour in skin of left thigh and buttock and above umbilicus	Present	Myelomatosis of bones and muscles
Hayes *et al.* (1952) ..	—	—	Biopsy of a cutaneous nodule mentioned	—	Myelomatosis with pancreatic tumour (P.M.)
Snapper *et al.* (1953) ..	65	F	Many firm nodules up to size of plum in skin of legs and trunk terminally	Absent	Myelomatosis of bones, liver, spleen, lymphoid tissue, diaphragm, epicardium, lungs, and muscle (P.M.); W.B.C. 3,450/c.mm.; 1% plasma cells
Kirkpatrick (1954) ..	43	F	Numerous cutaneous tumours on trunk and neck	Absent	Myelomatosis of bones; presented in both breasts

P.M. = Diagnosis confirmed at necropsy.

with treatment to the rapid or slow development of myelomatous lesions throughout the skeleton and other sites (Hellwig, 1943; Ritz and Meyer, 1952). Of the 16 cases of cutaneous plasmacytomata summarized in Table I only two originated in the nasopharynx (Ringertz, 1938; Saltzman and Borgström, 1950), the latter case being similar to our Case 1. In only one case, that of Piney and Riach (1931), was there a plasma cell leukaemia.

In Case 1 osteolytic lesions developed within two years of the appearance of the nasal growth, but the total course of the disease was protracted over seven years. At first the bony lesions were typical of those seen in myelomatosis, but later, despite a typical histological appearance, they showed an unusually pleomorphic radiological appearance with numerous, large, expanding tumours mainly confined to the ends of the long bones, and without diffuse marrow infiltration. In Case 2 the course was more rapid and the bony involvement minimal. As a general rule, extramedullary plasmacytomata and the lesions of myelomatosis respond well to irradiation early in the illness, but are later more resistant. There was a striking but transient sensitivity to various forms of treatment in both our cases: Case 1 responded well to 10 courses of radiotherapy, in addition to radium pack, nitrogen mustard, and urethane, but terminally there was no improvement with urethane, pentamidine isethionate, or "R.48."

Although gross extraskeletal tumours are unusual in myelomatosis, microscopical deposits are more commonly observed, particularly in lymph nodes, liver, and spleen (Cappell, 1929; Churg and Gordon, 1942; Lichtenstein and Jaffe, 1947; Limarzi, 1951), other sites being rarely affected.

Apart from direct local spread there is no universal agreement on whether the disease is of multifocal origin or disseminated by metastases. Shapiro and Watson (1953) suggested that myelomatosis is a generalized disease of haemopoietic tissues, and Lowenhaupt (1945) considered it to be a diffuse lesion of the entire reticulo-endothelial system. Certainly the lesions in myelomatosis appear to have a multifocal origin in the marrow itself (Cappell, 1951), and autochthonous growth may well explain the deposits observed in such potentially haemopoietic tissues as liver, spleen, and lymph nodes.

On the other hand, metastatic blood-borne dissemination offers an alternative origin for the growths found in the lungs, skin, heart, kidneys, and elsewhere. This view is favoured by the findings of Diggs and Sirridge (1947), who observed small numbers of plasma cells in the blood in as many as 27 of 53 cases. In addition, plasma cell leukaemia occasionally develops in myelomatosis. Had we found plasma cells in the blood stream of our two cases, as did Piney and Riach (1931), we should feel more confident that metastatic dissemination explains the numerous skin deposits, but until the principles underlying metastases of malignant reticuloses are better understood we prefer to accept Willis' (1953) view that both autochthonous growth and blood-borne dissemination occur in myelomatosis.

Summary

Two cases of myelomatosis are described, each of which presented with an extramedullary plasmacytoma of the upper air passages and later developed numerous widespread cutaneous deposits. One showed unusual osteolytic tumours, while in the other the predominant feature was a widespread involvement of the lymphoid tissue with isolated deposits in bone, heart, and stomach. Both differed in important respects from classical multiple myeloma and solitary plasmacytoma.

The recorded examples of cutaneous myelomatosis are briefly reviewed.

It is probable that the extraskeletal lesions in multiple myelomatosis are both multifocal and metastatic in origin.

We are much indebted to Professor D. F. Cappell and to Professor I. G. W. Hill for their criticisms; to Dr. D. C. Wilson and Mr. I. MacGregor Seex for permission to publish and to Dr. H. J. R. Kirkpatrick for the tissues from Case 1; to Dr. J. A. W. McCluskie and Dr. F. T. Land for permission to publish Case 2.

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GLYCOGEN STORAGE IN THE LIVER IN DIABETES MELLITUS

BY

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In this clinic, where 800 patients are seen, we have noted what appears to be a new syndrome. It occurs in young, severe, " brittle " diabetics, and is characterized by two predominant features—an enlarged liver found to be full of glycogen and a constant tendency to hypoglycaemia while taking insulin. The enlarged liver, not unusual in diabetes mellitus and commonly supposed to be due to deposition of fat, was in each case found by repeated biopsy to be distended with masses of glycogen, as in von Gierke's disease.

A survey of the literature indicates that the general consensus of opinion regarding the amount of glycogen in the liver in diabetes mellitus is that in this condition the amount is decreased (Mirsky, 1942; Korenberg, 1943), and observations on experimentally produced diabetes in animals by many authors (Cruickshank, 1913, to Bodo, Co Tui, and Farber, 1933) support these findings. Pemberton (1925) records the low figure of 0.04% glycogen in the liver in a severe diabetic dying of insulin hypoglycaemia. Vallance-Owen (1952), on the other hand, describes a young diabetic dying in severe ketosis and coma, whose liver at necropsy was distended with glycogen.

The use of " glucagon," the hyperglycaemic glycogenolytic factor said to be liberated in the alpha cells in the pancreas and the gastric mucosa (Sutherland and de Duve, 1948), has led to some interesting speculations. For instance, Kirtley, Waife, and Peck (1953a) and Kirtley, Waife, Helmer, and Peck (1953b) suggest that the smallness of the rise in the blood sugar (after the exhibition of " glucagon ") may be due to lack of liver glycogen in a patient with little or no endogenous insulin, i.e., the young, severe diabetic. Our own results in diabetic patients whose livers are full of glycogen are not in keeping with these observations.

We have found no evidence in the literature of a syndrome such as we describe, whereby severe diabetic patients exhibit hepatomegaly due to glycogen storage and yet are prone to hypoglycaemic attacks, presumably due to the fact that glycogen is not available to them for gluconeogenesis. In fact in a series of 50 cases of diabetes mellitus with homologous serum jaundice, liver biopsy was done during the acute phase and one or two years afterwards in order to determine any abnormal liver histology, and no comparable amounts of glycogen were found in either series. The distribution of glycogen in these livers and in our present series was identified and visually assessed by using the periodic-acid-Schiff reaction (P.A.S.) before and after digestion with diastase (Pearse, 1953).

In this respect our cases resemble von Gierke's disease, the important difference being the fact that patients in this series are proven severe or brittle diabetics and do not fulfil the criteria for the recognition of von Gierke's disease, as laid down by Ellis and Payne (1936). An attempt has been made to mobilize the excess liver glycogen by administering glucagon to our cases. Hubble (1954) has already demonstrated that this substance is unable to mobilize glycogen from the liver in von Gierke's disease, and our own analyses, which have followed closely the technique previously published by Kirtley *et al.* (1953a and b), suggest that for some reason, at present unknown, glycogenolysis is impaired in these patients—a feature already suggested by their tendency to hypoglycaemia.

Case Records

Case 1.—A. W. was a female nursery assistant, aged 25, who had had diabetes since the age of 13 (1941). Stabilization had always been difficult owing to recurrent attacks of hypoglycaemia; she was finally admitted to this hospital in August, 1953, complaining of recurrent swelling of the parotid glands, repeated attacks of hypoglycaemia, and amenorrhoea. While in hospital she noticed increasing abdominal distension and a feeling of epigastric discomfort; examination of the abdomen revealed a smooth, soft enlargement of the liver, three fingerbreadths below the right costal margin. Biopsy showed the hepatic cells everywhere to be distended with glycogen. Pain, swelling, and tenderness

over both parotid glands were at first thought to be mumps (there had been a recent history of contact in the nursery). However, these glands have fluctuated in size up to the present time and there appears to be some relationship to the hypoglycaemic attacks.

The diabetes may be regarded as brittle in type because with a morning fasting blood sugar of 500 mg.% the patient tended to develop typical hypoglycaemia (blood sugar less than 50 mg.%) later the same afternoon. The hypoglycaemia was always relieved by the administration of oral or intravenous glucose. For 10 months she had had amenorrhoea, but pelvic examination and curettage revealed no abnormality and in fact her menstrual cycle became normal later. Radiographs of the gastro-intestinal tract, chest, skull, and parotid glands were normal.

The hypoglycaemia became gradually more severe and abdominal pain and distension increased, and after surgical consultation it was decided to explore the abdomen on April 23, 1954, in the remote chance of finding a gross pancreatic lesion, although in a recent case of insulin adenoma seen by us the liver glycogen has been found to be microscopically normal. A smooth enlargement of the liver was confirmed, but nothing macroscopically abnormal was found in the pancreas although, as is shown below, the islets were small and scanty.

Case 2.—R. T. was a schoolboy aged 14, who had had diabetes since the age of 8 (1948). Stabilization presented the same difficulties as in Case 1 and he was finally admitted to this hospital in July, 1953, because of the sudden onset of abdominal distension, which was found to be due to enlargement of the liver, and in addition attacks of hypoglycaemia were now frequent. He also had well-marked insulin atrophy.

Biopsy showed the liver cells to be distended with glycogen. The abdominal distension persisted, some pyrexia and vomiting developed, and the diagnosis of a tuberculous peritonitis was entertained, especially as the Mantoux test was positive 1 in 10,000. Streptomycin and P.A.S. were given for a time but discontinued when the meteorism and pyrexia had subsided. A repeat biopsy showed that the liver remained full of glycogen and that it was not altered by the administration of thyroid extract, although the blood sugar remained at a higher level while on this glycogenolytic drug.

Case 3.—J. C. was a schoolgirl aged 13, who had had diabetes since the age of 11 (1951). From the first she had been remarkably unstable and required large doses of insulin. She was admitted to this hospital in November, 1953, with tonsillitis and her mother related recent attacks of hypoglycaemia as a new feature. On examination follicular tonsillitis, a distended abdomen, and an enlarged liver were found. Biopsy again showed a normal liver pattern with hepatic cells swollen with glycogen.

Stabilization was attempted with "lente" and "ultra lente" insulins, but repeated attacks of hypoglycaemia necessitated the use of another glycogenolytic agent—adrenaline—with a rise in blood sugar and partial control. Further liver biopsy after adrenaline revealed that the liver cells were still distended with glycogen.

Case 4.—K. C. was a schoolgirl aged 13, who had had diabetes since the age of 3½ (1942). She was admitted to hospital in January, 1954, because of hypoglycaemic attacks, distension of the abdomen, and enlargement of the liver. Liver biopsy showed architecture of the normal pattern, but distension of the cells with glycogen. Recurrent abdominal distension and epigastric pain were again prominent features as in the other cases. The use of adrenaline brought about some partial control of the hypoglycaemia as in the previous case, but liver biopsy once again revealed that the appearance of the glycogen-distended liver cells had not altered. An E.C.G. showed no abnormality.

Table I summarizes the histological and biochemical investigations which were performed on these patients. From the histological point of view it can be seen that, although the livers are full of glycogen, their architecture is normal. From the

TABLE I
HISTOLOGICAL AND BIOCHEMICAL FINDINGS

Investigations	Case 1 A. W.	Case 2 R. T.	Case 3 J. C.	Case 4 K. C.
No. of biopsies	2	4	2	3
Glycogen in liver	Excess	Excess	Excess	Excess
Liver pattern	Normal	Normal	Normal	Normal
Glycogen in muscle	"	—	—	"
Pancreas	Small and scanty islets	—	—	—
Gastric analysis	Hyper-chlor-hydria	—	Normal	Normal
Serum cholesterol (in mg.%)	250	—	220	300
17-Ketosteroids (mg.)	17	—	—	—
Glucose tolerance test (in mg.%)	490, 650, 500, 550, 650	100, 194, 352, 420, 364	510, 520, 540, 600, 540	444, 744, 696, 558, 508
Lactose tolerance test (in mg.%)	390, 420, 510, 590, 580	—	640, 660 720, 740, 700	400, 570, 600, 620, 600
Insulin tolerance test	Insensitive	—	Normal	Insensitive
Glucagon sensitivity (intravenous drip, mg.%)	425, 460, 475, 510	420, 440, 420, 420	—	450, 420, 480, 485
Serum inorganic phosphate with insulin (in mg.%)	Fell 4 → 3·6	Fell 2·3 → 2·38 → 2·43 → 1·48	2·3	Fell 5·3 → 5·1

fall in serum inorganic phosphate levels it is to be inferred that insulin activity is normal. The serum cholesterol levels and the lactose tolerance test were done to exclude von Gierke's disease. The insulin tolerance test was done to exclude insulin adenoma for which, in fact, a very careful visual search was made in Case 1.

Discussion

When we first recognized the existence of this syndrome we were greatly puzzled by the apparently conflicting nature of its component parts. The

four patients described in this paper were obviously unique when compared with the other diabetics, numbering about 800, at present attending the Clinic. Such features as hepatomegaly due to glycogen storage, associated with severe hypoglycaemic tendencies, in patients who were also severe or brittle diabetics constituted a problem for which we have as yet found no entirely satisfactory solution. Enlargement of the liver constitutes one of the most significant features of the syndrome. We are, of course, familiar with enlargement of the liver in diabetic patients associated with deposition of fat or haemochromatosis, but the discovery of glycogen as the cause of hepatomegaly in these patients is very difficult to explain. We suggest that there are two possible explanations.

(1) All our cases were exceptionally severe or brittle diabetics in whom stabilization had been virtually impossible. For years their blood sugar had been elevated well above the normal levels, interrupted at frequent intervals by repeated attacks of hypoglycaemia. Bouckaert and de Duve (1947) have stated that the normal regulator of sugar metabolism is the blood sugar itself and that upon it the effect of insulin depends. Apparently glycogen storage in the liver will occur in the depancreatized animal as a result of hyperglycaemia (Bodo *et al.*, 1933) and thus it would appear that insulin acts as a catalyst in carbohydrate metabolism, allowing the tissues to achieve at normal blood sugar levels that for which high levels are required in its absence (Vallance-Owen, 1952). By virtue of the severity of the diabetes in the patients whom we have described, the accumulation of glycogen may have resulted from prolonged periods of hyperglycaemia.

(2) The only other condition in which glycogen is stored in the liver in comparable amounts to that in this syndrome is von Gierke's disease (glycogen storage disease). This is a congenital disturbance possibly inherited as a Mendelian recessive character, in which there appears to be a defect of the glycogen-splitting enzyme, which produces an abnormal stability of glycogen. According to Crawford (1946), in von Gierke's disease the normal reaction glucose→glycogen→glucose is replaced by the sequence glucose→fat→ketone bodies and glucose, presumably due to the lack of the normal enzyme reaction. We wish to make it quite clear that our cases are not examples of von Gierke's disease. We have utilized the criteria laid down for the diagnosis of this disease by Ellis and Payne (1936) and have demonstrated that our patients do not satisfy these requirements (Table I). Briefly, our patients do not have a low fasting blood sugar level, they are confirmed severe diabetics, and their hypoglycaemic tendencies are partially controlled by the administration of thyroid extract or adrenaline. Further, the blood cholesterol levels are not significantly raised. Nevertheless, hypoglycaemic tendencies and an enlarged liver are features closely resembling two major components of von Gierke's glycogen storage syndrome. In this connexion we may find the explanation for the recurrent attacks of hypoglycaemia which all our patients have shown. Because they are all severe diabetics, they require daily insulin to stave off diabetic pre-coma. If we postulate that they lack the glycogen-splitting enzyme and that their liver glycogen is abnormally stable, as in von Gierke's disease, the recurrent hypoglycaemic episodes from which all of them suffer may be due to the virtually unopposed action of the daily insulin injections. Some support for this theory is found in Case 1 (A.W.),who became hypoglycaemic at regular intervals twice daily, and on each occasion it could be inferred that insulin activity was maximal at the time. Conversely her morning fasting blood sugar was invariably in the region of 500 mg.%.

To establish this syndrome as a separate entity, we have pointed out that, although it bears a certain resemblance to von Gierke's disease, our patients do not satisfy the diagnostic criteria already indicated. It is important also to stress that the syndrome differs greatly from the more easily defined picture which we refer to as diabetes mellitus. The ordinary diabetic patient, however severely affected, can be stabilized by appropriate diet and insulin therapy. Diabetic neuropathy, renal lesions, retinopathy, are all recognized associated features of the diabetic syndrome, and liver enlargement, when it occurs, is always due to fatty change or occasionally haemochromatosis. In the present series of patients all attempts at stabilization failed despite frequent variations of diet and the use of all types of insulin therapy. Further, the association of acute attacks of abdominal pain, meteorism, and liver enlargement (due to glycogen storage), together with recurrent attacks of hypoglycaemia which cannot be controlled by modification of insulin therapy, are never found in the ordinary diabetic patient. The response of our patients to "glucagon" (H.G.F.) has greatly interested us. From Table I it may be seen that the rise in blood sugar following an intravenous drip containing this substance was surprisingly small, when it is recalled that the liver cells of all these patients are distended with masses of glycogen. This apparent inability of "glucagon" to mobilize glycogen from the liver in any significant amount is similar to results published

by Hubble (1954) in von Gierke's disease. Alternatively, Kirtley *et al.* (1953a and b) suggest that in the ordinary severe diabetic the smallness in the rise of blood sugar following " glucagon " is due to lack of liver glycogen in these patients. We can only assume that in our series of cases glycogenolysis is impaired, a feature already suggested by their tendency to hypoglycaemia.

In conclusion we would refer to Case 1 (A. W.) in which, after prolonged discussion, laparotomy was considered justified. Although biochemical investigation did not suggest the presence of an insulin adenoma, we were of the opinion that in such a bizarre syndrome as this only direct inspection of the pancreas would satisfy us that no gross pancreatic lesion existed. As stated above, the pancreas appeared normal on inspection, but histologically we observed that the islets were remarkably small and extremely scanty. These changes could be secondary to prolonged hyperglycaemia (Vallance-Owen, 1952), as the patient had never achieved stabilization during a diabetic life of 12 years.

We hope that in this paper we have justified the introduction of a new syndrome, which should be sought in any young, brittle diabetic patient with enlargement of the liver.

Summary

In a diabetic clinic of 800 patients a new syndrome has been described in four cases having a brittle type of diabetes mellitus, associated with enlargement of the liver due to glycogen storage and recurrent attacks of hypoglycaemia.

The cases are described from both clinical and biochemical aspects.

The nature of these findings is discussed with particular reference to von Gierke's disease, the use of " glucagon " (H.G.F.), and the more common syndrome of diabetes mellitus.

We are deeply grateful to Dr. W. R. Kirtley, of Eli Lilly & Co., not only for the generous supply of "glucagon" but also for information about the technique of using it.

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CYTOLOGICAL EXAMINATION OF SPUTUM IN RELATION TO ITS MACROSCOPIC PURULENCE

BY

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Investigations into the bacteriology of chronic bronchitis (May, 1953a and b) have shown that purulent sputum usually contains one or more pathogens and that such a sputum can be rendered mucoid, at any rate temporarily, when the patient is treated with the appropriate antibiotic. Subsequent studies (May, 1954) included patients diagnosed clinically as infected asthma, and the macroscopical appearance of the sputum from these patients suggested the presence of pus, although in many instances pathogenic organisms could not be demonstrated and chemotherapy failed to alter its appearance. Examination of the cellular contents of such sputa showed that in most cases the "pus" consisted mainly of eosinophils. As a result of these observations investigations have been undertaken to develop a satisfactory method for the cytological examination of sputum, for the distinction between neutrophil and eosinophil "pus" is clearly of great importance.

The examination of single smears of sputum for eosinophils is of little value, since the distribution of these cells is often irregular (see below). The sputum must, therefore, be homogenized in order to obtain an even suspension of cells before the film is made. Pancreatin used for this purpose (Rawlins, 1953) does not cause cellular destruction.

This paper describes a method whereby the total number of cells per unit volume of sputum can be estimated as well as the relative number of eosinophils.

Method

Preliminary Washing of the Sputum.—In order to free the sputum as far as possible from adherent saliva it is first rinsed in about five times its volume of physiological saline solution, which is then removed by means of a Pasteur pipette.

Liquefaction of the Sputum.—The specimen is liquefied by the addition of 1% pancreatin solution buffered to *p*H 7.6 (Rawlins, 1953). A measured volume of the solution is added to approximately an equal volume of sputum; the mixture is thoroughly shaken and is then incubated in a water-bath at 37° C. until liquefaction is complete. The total volume of the liquefied sputum is measured, and, knowing the volume of pancreatin used, the dilution of the sputum is calculated.

The Total Cell Count.—A drop of the liquefied sputum, previously well shaken to ensure uniform suspension of the cells, is placed in a Neubauer haemocytometer and the cells are enumerated by the standard methods applicable to cell counting in other fluids. In most instances no additional dilution of the sputum is required, but occasionally, for exceptionally cellular specimens, the sputum may require further dilution before accurate counting is possible.

The Differential Cell Count.—A film of the liquefied sputum is dried on a microscope slide, fixed in methyl alcohol, and stained with haematoxylin and eosin or Leishman's stain. A representative number of cells (100 are usually sufficient) are counted under the microscope and the relative number of each type of cell determined. In this investigation interest centred particularly on the eosinophils in sputum and various staining methods, both wet and dry, were tested. Haematoxylin and eosin gave the most consistently satisfactory results, though Leishman was often almost as good. In some sputa in which most of the cells were degenerate haematoxylin and eosin was definitely superior, and this method has been adopted for routine use.

Results

Distribution of Eosinophils in Unliquefied Sputum.—Table I shows examples illustrating the irregular distribution of eosinophils which may be encountered in macroscopically purulent sputum.

Survival of Leucocytes in Sputum Liquefied by Pancreatin.—In order to demonstrate that pancreatin had no lytic effect on cells in sputum, 12 specimens were liquefied and total cell counts performed immediately after liquefaction and again after

TABLE I

UNEVEN DISTRIBUTION OF EOSINOPHILS AND NEUTROPHILS IN PURULENT SPUTA*

Sputum	Film	Percentage of Polymorphs	
		Eosinophils	Neutrophils
1	1	86	14
	2	8	92
2	1	57	43
	2	10	90
3	1	73	27
	2	4	96
4	1	41	59
	2	28	72

* Films were made from different parts (all macroscopically purulent) of each sputum. They were stained with haematoxylin and eosin and differential counts of the types of polymorphs were made. The total number of cells counted in each smear was 500. The figures in the table show the variations in the proportions of eosinophils and neutrophils in films from two different parts of each specimen.

24 hours of standing at room temperature. Within the limits of experimental error for the counting technique the same counts were obtained for each pair of observations.

The possibility that pancreatin might destroy cells during the period in which liquefaction was taking place could not be investigated in sputum, owing to the impossibility of estimating the number of cells present before liquefaction. However, no diminution in the cell count, apart from that caused by the dilution when pancreatin was added, was found when purulent pleural fluids were incubated with pancreatin, suggesting that the cells in sputum were also unlikely to be destroyed.

Relationship between Total Cell Counts and Macroscopic Appearances of Sputum.—Enumeration of the total cells has been carried out in 602 specimens of sputum. The relationship between the counts and the macroscopic appearances of the specimens is shown in Table II.

TABLE II

MACROSCOPIC APPEARANCE AND TOTAL POLYMORPH CONTENT OF 602 SPECIMENS OF SPUTUM

Macroscopic Appearance	No. of Specimens	No. of Cells (per c.mm.)	
		Range*	Mean†
Thick pus	53	7,000–89,000	32,600
Pus and mucus (predominantly pus) ..	182	1,500–50,000	15,700
Pus and mucus (predominantly mucus)	162	500–8,000	2,686
Mucus	205	38–3,000	837

* "Range" = extreme limits of cell counts in each type of sputum.
† "Mean" = the mean of the counts of all the specimens in each class of sputum.

Discussion

Routine determination of the number and nature of the cells in sputum presents difficulties, and it is not suggested that the complete total and differential counting method described here should be employed in every patient. Estimation of the total number of cells per unit volume of sputum has very limited practical value. As will be seen from Table II the correlation between macroscopic purulence and the cell content of sputum is very poor. This is understandable in the light of the observation of Elmes and White (1953) that desoxyribonucleoprotein (D.N.P.) fibres, derived from the breakdown of polymorphs, are often responsible for much of the macroscopic purulence of sputum. Elmes and White state that in the early stages of acute exacerbations of chronic bronchitis polymorphs are exceedingly numerous in the sputum, but later their number falls and D.N.P. fibres increase. Clearly, therefore, day-to-day enumeration of the cells in the sputum would provide no guide to the progress of this type of patient. In patients with chronic bronchitis who have no acute exacerbation, however, the cell content of the sputum has been found in this laboratory to remain relatively constant from day to day before treatment, and changes in the cell counts in these patients have been useful in assessing the results of treatment. Mention of the use of the total cell-counting technique for this purpose was made in an earlier report by May (1953b).

Although enumeration of the total cells in sputum on one occasion only has little practical value, the differential count is important. For routine purposes a count is probably unnecessary, but a film should always be stained and the cells examined in order to be certain that apparent "pus" is really composed of neutrophils and not mainly of eosinophils. Owing to the irregular distribution of eosinophils in some specimens of sputum, especially those containing neutrophils also (Table I), a single smear from the unliquefied sputum is unreliable. Mechanical methods of homogenization, such as shaking with glass beads, are liable to destroy the cells, and some form of enzymatic digestion is therefore required. The use of pancreatin is very satisfactory.

The techniques described in this paper were evolved as part of a study of the chemotherapy of chronic respiratory infections. It is possible that liquefaction by pancreatin may also be valuable in the search for malignant cells in sputum, since after liquefaction the cells can be concentrated by centrifugation, thus allowing a far greater number to be examined than is possible when smears from unliquefied sputum are used.

Summary

The distinction between neutrophils and eosinophils in macroscopically purulent sputum is important, since the "pus" in some instances is composed mainly of eosinophils.

A method is described for enumerating both the total cells per unit volume of sputum and the proportion of eosinophils and neutrophils. Since eosinophils are often irregularly distributed in the sputum preliminary homogenization by the action of pancreatin is required.

I wish to thank Dr. J. R. May for his help and advice in the preparation of this article.

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THE DRIED DISC TECHNIQUE WITH BACITRACIN, CARBOMYCIN, ERYTHROMYCIN, POLYMYXIN B, AND TETRACYCLINE

BY

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The bacteriological control of antibiotic therapy is becoming increasingly important in view of the steady production in the U.S.A. of potent new antibiotics, some of which have a limited range of activity. It is therefore necessary to have a rapid and reliable method of carrying out the sensitivity tests in order to make this a practical routine procedure.

The dried disc technique gives satisfactory results with penicillin, streptomycin, chloramphenicol, chlortetracycline (aureomycin), and oxytetracycline (terramycin) and is now widely used. It is therefore desirable that the range of discs should be extended to include bacitracin, carbomycin (magnamycin), erythromycin, polymyxin, and tetracycline.

Technique

The preparation of the dried discs followed closely the technique with the established antibiotics (Fairbrother and Martyn, 1951). Discs (8 mm. diameter) were punched from coloured blotting paper (Ford) and sterilized for one hour in the hot air oven at 150° C. The discs were then carefully impregnated with one drop of freshly prepared solutions of the different antibiotics, using a "50-dropper" pipette, and then dried for one and a half to two hours in the incubator, after which they were stored in sterile screw-cap bottles (1 oz.) at 0° to 4° C. (Table I).

TABLE I
PREPARATION OF THE DRIED DISCS

Antibiotic	Colour of Disc	Strength of Solution per ml.	Final Amount on Disc
Bacitracin ..	Blue	500 units	10 units*
Carbomycin ..	Grey	500 μg.	10 μg.
Erythromycin ..	Red	500 ,,	10 ,,
Polymyxin B ..	Yellow	25,000 units	500 units†
Tetracycline ..	Purple	500 μg.	10 μg.

* 1 mg. (bacitracin) = 50 units (approx.)
† 1 μg. (polymyxin) = 7–10 units.

Plates of appropriate media (agar, blood-agar, or chocolate-agar) were seeded either by flooding with a young broth culture or with the platinum loop to give a heavy, uniform, and pure growth. The discs were then added at regular intervals and readings were made after incubation at 37° C. for 18 hours. In the case of rapidly growing bacteria, readings can often be made earlier. For comparative purposes, a zone of inhibition of 14 mm. in diameter was considered to indicate sensitivity to the particular antibiotic.

The final strength of the various antibiotic solutions was determined by previous experience with the standard antibiotics and also by direct comparison with the results given by the serial dilution technique; consideration was also given to the tissue levels resulting from routine therapeutic practice.

Results

The new antibiotics differ considerably in their range of antibacterial activity, and in consequence they have been arranged arbitrarily, for convenience of discussion and comparison with established antibiotics, into three groups: (1) erythromycin, carbomycin, and bacitracin—acting mainly on Gram-positive cocci; (2) tetracycline with a wide range of activity; (3) polymyxin B—effective against some Gram-negative bacilli, in particular *Ps. pyocyanea*.

A wide variety of organisms was included in the test series; all were active pathogens freshly isolated from infective processes. Initially the new antibiotics were added to duplicate plates of the test organism, but later all discs were added to the same plate. This procedure, involving seven or eight discs per plate, proved satisfactory provided the discs were carefully spaced. It is now the routine practice.

Group I.—Carbomycin was originally included in this series, but it was eventually omitted, as unsatisfactory clinical trials were reported in the U.S.A. (Finland, 1953). The results are presented

together with those given by penicillin, which has a comparable antibacterial spectrum (Table II). In all cases the various antibiotics were tested simultaneously against the individual organisms.

TABLE II
SENSITIVITY OF COMMON PATHOGENS TO ERYTHROMYCIN, CARBOMYCIN, BACITRACIN, AND PENICILLIN BY DISC TECHNIQUE

Organism	No. Tested	Percentage Sensitive			
		Penicillin	Erythromycin	Bacitracin	Carbomycin
Staph. aureus ..	300	43	99·7	100	100 (129 tested)
Strep. haemolyticus (Group A) ..	32	100	100	100	
Strep. haemolyticus (other groups) ..	25	100	100	100	
Strep. faecalis ..	65	13·8	100	100	
Pneumococcus ..	17	100	100	100	
Coliforms	110	0	0	0	
Proteus	80	0	0	0	
Ps. pyocyanea ..	70	0	0	0	
H. influenzae and *H. parainfluenzae* ..	35	34	100	0	
Monilia albicans ..	15	0	0	0	

Erythromycin, carbomycin, and bacitracin proved remarkably effective against the Gram-positive cocci, irrespective of their reaction to penicillin; sharply defined zones of inhibition were produced. Several strains of *Staph. aureus*, isolated from unrelated infections, proved resistant to the established antibiotics but sensitive to erythromycin and bacitracin. At the end of this series, a resistant strain of *Staph. aureus*, isolated from an infected wound, gave a clear zone of inhibition with bacitracin and only an indefinite zone with erythromycin. Tests were consequently carried out with individual colonies from the primary culture; the erythromycin discs gave a slight and indefinite zone of inhibition and the organisms were only inhibited by 8 to 64 μg./ml. in the serial dilution test.

Erythromycin, but not bacitracin, was also effective against the influenza and parainfluenza bacilli. The coliform, *Proteus*, *Ps. pyocyanea*, and *Monilia* strains invariably proved resistant.

Group II.—Tetracycline has a chemical structure closely related to chlortetracycline (aureomycin) and oxytetracycline (terramycin), from which it was originally prepared. A similar, but not identical, antibacterial spectrum has been reported and differences in stability and toxicity have been observed (Finland, 1953). These products are closely related and have been grouped together.

Stability tests on solutions of the three antibiotics (128 μg./ml.) were carried out at 4° C. and room temperature. In both series chlortetracycline proved to be least stable, with oxytetracycline slightly more stable than tetracycline.

Disc sensitivity tests were carried out under strictly comparable conditions on a number of common pathogens; the results are given in Table III.

The results indicate that these analogues have a comparable, but not identical, range of antibacterial activity. Some bacteria, particularly gram-negative bacilli, exhibited marked differences in their reaction to the three compounds when tested by the disc techniques. These results were confirmed, in a number of instances, by the serial dilution tests.

TABLE III
SENSITIVITY OF COMMON PATHOGENS TO CHLORTETRACYCLINE, OXYTETRACYCLINE, AND TETRACYCLINE BY DISC TECHNIQUE

Organism	No. Tested	Percentage Sensitive		
		Chlortetracycline	Oxytetracycline	Tetracycline
Staph. aureus ..	250	92	92	92
Strep. haemolyticus (Group A) ..	32	100	100	100
Strep. haemolyticus (other groups) ..	25	100	100	100
Strep. faecalis ..	60	60	61	61
Pneumococcus ..	17	100	100	100
Coliforms	105	84	92	85
Proteus	75	10·8	10·8	9·3
Ps. pyocyanea ..	70	4·3*	15*	2·8*
Friedländer's bacillus	8	75	100	75
H. influenzae and *H. parainfluenzae* ..	32	100	100	100
Monilia albicans ..	15	0	0	0

* = Some strains slightly sensitive (zone < 14 mm.)

Group III.—Although the polymyxins, in particular aerosporin, were isolated some time ago, they have not been widely used for therapeutic purposes because of their toxic effects. The toxicity has been reduced and, as infections with *Ps. pyocyanea* do not respond readily to the other antibiotics, there is now an increasing demand for polymyxin B, particularly for local application.

A number of tests have been carried out and the results are compared with those given by streptomycin and chloramphenicol under strictly comparable conditions (Table IV).

TABLE IV
SENSITIVITY OF SOME PATHOGENS TO STREPTOMYCIN, CHLORAMPHENICOL, AND POLYMYXIN B BY DISC TECHNIQUE

	No. Tested	Percentage Sensitive		
		Streptomycin	Chloramphenicol	Polymyxin B
Strep. faecalis ..	60	5	71	0
Coliforms	70	80	90	90
Proteus	75	73	76	0
Ps. pyocyanea ..	70	17	13*	98
H. influenzae and *H. parainfluenzae* ..	30	100	100	100
Monilia albicans ..	15	0	0	0
Cryptococcus neoformans	1	0	0	100

* = Some strains slightly sensitive (zone < 14 mm.)

These results indicate clearly the effectivenesss of polymyxin against *Ps. pyocyanea*; by the tube technique, it was found that 65 out of 66 strains were inhibited by concentrations of 4 to 16 units/ml., and one out of 66 strains by 128 units/ml. While these concentrations are relatively high, many of these strains were isolated from superficial lesions, such as infected burns and wounds as well as chronic otitis, in which direct application of the antibiotic is readily accomplished.

It is interesting to note that, while 15/15 strains of *Monilia* were resistant to all antibiotics, a strain of *Cryptococcus neoformans*, isolated from a fatal infection, proved to be resistant to all antibiotics except polymyxin, which gave a clear zone of inhibition by the disc technique and a tube sensitivity of 16 units/ml.

Discussion

The dried disc technique has proved a satisfactory routine method of determining the sensitivity of the common pathogens to the new antibiotics. It is a rapid qualitative test which provides useful information to guide the selection of a potentially effective product for therapeutic purposes.

The best results are given by pure cultures prepared from the primary culture of the infected material, but under certain circumstances, particularly when a heavy pure growth seems probable, useful information may be obtained by the addition of the discs to the primary culture. A duplicate plate should always be used for this purpose. Whenever equivocal results are given, further tests should be done with the serial dilution technique.

Erythromycin and bacitracin are very effective against gram-positive cocci and they should be especially useful in the treatment of infections caused by penicillin-resistant staphylococci. Caution is, however necessary, as bacteria tend readily to develop resistance to erythromycin; several resistant strains of *Staph. aureus* have been isolated since the completion of this series.

Tetracycline has a wide range of activity, which is similar, but not identical, to that of the other tetracycline compounds, aureomycin and terramycin. Tetracycline must therefore be treated as a distinct antibiotic and not merely as a substitute for established products.

Polymyxin B proved very effective against *Ps. pyocyanea* and also against one strain of *Cryptococcus neoformans*. It is particularly useful for direct local application.

Summary

The dried disc technique has proved a useful method of testing the activity of erythromycin, bacitracin, carbomycin, tetracycline, and polymyxin B. It has given satisfactory results with a wide range of common pathogens. The results have been compared with those given by the standard antibiotics.

We wish to thank Messrs. Lederle Laboratories Division, Pfizer, Ltd., Eli Lilly & Co., Ltd., and Abbotts Laboratories, Ltd., for special supplies of tetracycline (achromycin), carbomycin, erythromycin (ilotycin and erythrocin) respectively; also Dr. J. Davson for the culture of *Cryptococcus neoformans*.

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SENSITIVITY TESTS FOR THE POLYMYXINS

BY

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Although the value of polymyxins B and E in the treatment of infections with Gram-negative bacilli is well recognized, they are not usually used unless the causal organisms are insensitive to antibiotics with wider activity. In consequence, sensitivity tests for them are not common laboratory procedures and it is not generally appreciated that the usual methods are not entirely satisfactory.

Judged from experience with the commoner antibiotics, organisms often appear insensitive to the polymyxins when tested by the agar diffusion method, yet the dilution (inclusion) method may show them to be fully sensitive. This latter method too can give very variable results, especially when a fluid medium is used, and on a solid medium the strain may often appear to consist of resistant and sensitive variants when in fact it is homogeneous. The discrepancies in both of these methods are probably due to the fact that the mode of action of the polymyxins differs from those of the more commonly used antibiotics, and studies of the action have revealed two important facts.

First, the polymyxins are always bactericidal (Brownlee and Bushby, 1948; Brownlee, Bushby, and Short, 1952) and bacteriolytic (White, Alverson, Baker, and Jackson, 1949; Bushby, 1952), apparently through being absorbed by the cell membrane and altering its permeability. They thus act in a manner similar to the cationic detergent, cetyltrimethylammonium bromide (Bushby, 1952; Few and Schulman, 1953).

Secondly, the minimum bactericidal concentration of the polymyxins depends upon the number of organisms present (Brownlee *et al.*, 1952) due to the fact that a certain quantity of polymyxin is expended in killing each organism. The amount absorbed is therefore roughly proportional to the sensitivity of the strain and to the number of organisms present (Bushby, 1952), but the relationship is complicated by the fact that complete saturation of the organisms is not necessary for the bactericidal effect. Incidentally the organisms do not have to be living for this absorption to occur.

From these facts it follows that it is not possible to state an exact sensitivity figure for any given organism without defining the size of the inoculum used in the test. Swift and Bushby (1951) showed that the apparent sensitivity of a strain of *Haemophilus influenzae* ranged from 0.8 units to 25 units per ml., depending upon the size of the inoculum.

The following experiments illustrate the poor correlation between the apparent sensitivities when measured by the diffusion and dilution methods. Three different-sized inocula of eight organisms of different genera were used.

Technique

Diffusion Method.—Filter paper discs 8 mm. in diameter were impregnated with 40 units and 100 units of polymyxin B per disc by adding 0.05 ml. of an aqueous solution of the antibiotic. Two discs, one of each concentration, were placed on nutrient agar plates infected with the three different-sized inocula. The inocula were from an 18-hour broth culture diluted to give growths of colonies that were heavy confluent, just confluent, and separate. Plates 9 cm. in diameter were used and 0.3 ml. of the diluted culture was added to 3 ml. of molten nutrient agar and poured over a base layer 3 mm. thick. After 18 hours' incubation at 37° C. the diameters of the zones of inhibited growth were measured, the measurement including the diameter of the disc.

Dilution Method.—Polymyxin B was added aseptically to molten nutrient agar in twofold increasing concentrations ranging from 0.15 to 160 units per ml., distributed in 5 ml. quantities into tubes and sloped.

By this procedure a number of sets of tubes containing graded concentrations of polymyxin were obtained and one set was used for each of the three inocula of the eight organisms. One loopful (3 mm. diameter) of diluted culture was spread over the surface of the medium, and the three dilutions of each organism were such that after 18 hours' incubation growth in the control tubes (without antibiotic) was of the same order as that of each of the three types obtained on the plates in the diffusion method. The concentration of polymyxin which inhibited 90% or more of growth, as compared with the control tube, was recorded.

Results

These are shown in Table I. The disc containing the higher concentration of the antibiotic was intended to produce conditions similar to those recommended by Thompson (1950), i.e., that moderately resistant, moderately sensitive, and fully sensitive organisms should show varying-sized zones of inhibition, and the disc with the lower concentration to fulfil the recommendations of Fairbrother and Martyn (1951), i.e., that only organisms sensitive to therapeutic concentrations of the antibiotic should show a zone of inhibition. For the latter 40 units were chosen because they produced no zone of inhibition with *Streptococcus pyogenes*. The table shows that it is only within narrow limits that the recommendations of Fairbrother and Martyn are achieved, for although by the deliberate arrangement of the concentration *St. pyogenes* can be made to appear insensitive, there is no correlation between the sizes of the zones of inhibition and the sensitivities of the more sensitive strains as shown by the dilution method. With the higher 100 unit concentration of polymyxin the correlation is no better, for although the diffusion method places *St. pyogenes* in the correct order of sensitivity when the small inoculum is used, with the heavier inoculum *St. pyogenes* appears to be more sensitive than *Bact. coli*.

TABLE I

RESULTS OF TESTS WITH POLYMYXINS B AND E

Organism	Dilution of Culture	No. of Colonies	Method of Serial Dilution in Nutrient Agar	Diffusion Method (Zone Diameter—mm.)	
			Minimum Concentration Inhibiting 90% Growth	40 Units per Disc	100 Units per Disc
Sh. paradysenteriae	10^{-1}	+++	2·5	13	16
	10^{-2}	++	0·6	15·5	18
	10^{-4}	+	0·3	17	23
S. typhosa ..	10^{-1}	+++	5·0	11	15
	10^{-2}	++	2·5	12	18
	10^{-4}	+	1·25	15	21
Bact. coli ..	10^{-1}	+++	5·0	12	13
	10^{-2}	++	2·5	13	16
	10^{-4}	+	0·6	14	18
Kleb. pneumoniae	10^{-1}	+++	5·0	Nil	14
	10^{-2}	++	2·5	11	18
	10^{-4}	+	2·5	11	19
Ps. aeruginosa	10^{-1}	+++	20·0	11	15
	10^{-2}	++	10·0	14	18
	10^{-3}	+	5·0	14	20
St. pyogenes	10^{-1}	++	50·0	Nil	14
	10^{-2}	+	50·0	,,	16
S. aureus ..	10^{-1}	+++	>160	,,	Nil
	10^{-2}	++	>160	,,	,,
	10^{-4}	+	>160	,,	,,
P. vulgaris ..	10^{-1}	+++	>160	,,	,,
	10^{-2}	++	>160	,,	,,
	10^{-4}	+	>160	,,	,,

+++=Heavy confluent. ++=Just confluent. +=Separate colonies.

Discussion

The failure of the diffusion method with the polymyxins can be attributed to three main causes. First, there is the difficulty of controlling the size of the inoculum; secondly, because of their large molecular size and highly non-specific absorbing properties the polymyxins diffuse very poorly; and thirdly, the area of the zone of inhibition varies with the rate of multiplication of the organism, because when there is an appreciable increase in the number of organisms before the slowly diffusing antibiotic reaches them, the effect is the same as would be obtained by using a heavier inoculum. Another factor which will also cause variations in the area of the zone is the continued absorption of the diffusing polymyxin by the killed organisms, and the larger the inoculum the more pronounced will be this effect. The importance of the size of inoculum in the diffusion method for the common antibiotics has, however, been stressed by Fairbrother and Martyn (1951), but they recommend using a heavy inoculum, which would be most misleading with the polymyxins.

In the experiments with the dilution method the level of polymyxin which inhibits 90% or more of the growth was taken as the minimum inhibiting concentration. This was done because the end-point is often not sharp, due to a few colonies growing in concentrations several times greater than that which kills most of the organisms, and it is these colonies which can give the impression that a culture is heterogeneous. Re-testing of these apparently less sensitive organisms shows them, however, to be no more resistant than the organisms killed by the lower concentrations, and their survival was presumably due to the fact that having escaped the action of the polymyxin, perhaps because they were in a clump, they then multiplied until they were too numerous for the concentration of residual unabsorbed polymyxin. The ability of organisms already dead to continue to absorb polymyxin would also add to this shielding effect. When a fluid instead of a solid medium is used the organisms that survive for the same reason will multiply and cause the strain to appear less sensitive than it really is.

That *St. pyogenes* should show some sensitivity may be surprising, for the polymyxins are usually regarded as being specifically active against the Gram-negative bacilli, with the exception of *Proteus vulgaris*, but it is unlikely that any organism is completely resistant. Small inocula of *St. pyogenes*

are sensitive to concentrations of 50 units per ml. and some strains of *Staphylococcus aureus* to 200–500 units per ml. *P. vulgaris*, in spite of its high resistance, is nevertheless killed by concentrations of 10,000 units per ml. Infections with these less sensitive organisms may be capable of being controlled by topical therapy with the polymyxins, but it is only those due to the more sensitive organisms that can respond to parenteral therapy. It is therefore important that the test should be sufficiently accurate to detect these differences. In spite of its limitations the dilution method can be standardized sufficiently to give fairly consistent and unequivocal results, and a recommended method for routine procedure, together with a comment on the standardization of the polymyxins, is given in the addendum.

Summary

Because the diffusion sensitivity method is unsatisfactory with the polymyxins, a serial dilution method in solid medium, using small inocula, is recommended, and the concentration of polymyxin which kills 90% of the organisms can be conveniently accepted as the minimum inhibitory concentration.

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ADDENDUM

Recommended Technique.—The polymyxin is added to molten nutrient agar, which is then sloped and inoculated with about 50 organisms. After 18 hours' incubation that concentration which inhibits approximately 90% or more of the growth is taken as the end-point. In practice, the medium can be sterilized after addition of the polymyxin by heating at 100° C. for 20 minutes and stored in the refrigerator for at least six months without significant loss of potency. Tubes containing 2, 5, 10, 25, and 50 units per ml. are usually sufficient for routine use. With most organisms one standard loopful (3 mm.) of a 10^{-5} dilution of an 18-hour broth culture is a suitable inoculum.

Standardization of the Polymyxins.—This may cause confusion, because the polymyxins are among the few antibiotics still standardized on a unit basis. The units are arbitrary ones and reference standards of known unitage are used by the manufacturers for assaying unknown samples. The purest samples of polymyxins B and E have contained approximately 10,000 units per mg., and as these were crystalline they may have been 100% pure. When the potency of polymyxin is expressed on a weight basis, as it is in the United States, it is assumed that there are 10,000 units per mg. or 1 unit = 0.1 μg. The polymyxin B available in this country is issued on a unit basis, and as it is usually about 70% pure it contains only about 7,000 units per mg.

For the sensitivity test the polymyxin can be taken from therapeutic material, either by dissolving a weighed sample in water or by dissolving the whole contents of the container in a known volume. In aqueous solution the polymyxins are stable for at least six months when kept in the refrigerator.

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FURADANTIN IN PROTEUS BACILLURIA

PRELIMINARY COMMUNICATION

BY

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The widespread use of antibiotics has increased the importance of resistant pathogens. Unmasked infections due to organisms such as *Candida* (Rankin, 1953), *Pseudomonas*, and *Proteus* (Yow, 1952) are presenting therapeutic problems. The search for new substances with which to treat such infections is therefore amply justified.

Nitrofurantoin is one substance which has been investigated in the U.S.A., and several reports on its efficacy have been published (Norfleet, Beamer, and Carpenter, 1952; Mintzer, Kadison, Shales, and Felsenfeld, 1953; Carroll and Brennan, 1954). Nitrofurantoin is N-(5-nitro-furfurylidene 1-amino-hydantoin). It is soluble in water to the extent of 18 mg. per 100 ml. at *p*H 7. It is administered orally. Forty per cent. of the administered dose is excreted in the urine (Mintzer *et al.*, 1953) and the rapidity of excretion necessitates six-hourly administration.

As a small supply of this substance became available in the form of "furadantin," it was decided to employ it in an attempt to assess its efficacy against *Proteus*. Cases of *Proteus* bacilluria were chosen, not because it was felt that such conditions are of outstanding gravity, but because the drug is largely excreted in the urine where it may be expected to exert a strong effect.

Material and Methods

Selection of Cases.—Cases selected were either patients who had undergone retropubic prostatectomy for benign prostatic hypertrophy whose catheter had been removed, or patients under treatment for post-prostatectomy stricture, or under hormone treatment for carcinoma of the prostate.

Cases of infections with strains of *Proteus* resistant to 5 mg. per ml. "furadantin" *in vitro* were rejected, though one such case served as a control in Group I.

Control cases were selected from similar groups in the same ward, and were treated with a high fluid intake only.

No attempt at random sampling was made. Cases were designated "test" and "control" arbitrarily.

Dosage.—The recommended dosage is 5–8 mg. per kg. body weight, which approximates to 100 mg. six-hourly in the weight range 170–250 lb. It was decided to use this standard dose for all our cases, and, since supplies of "furadantin" were limited, to treat cases for five days only.

One of our patients complained of diarrhoea on the fifth day of treatment. No other evidence of toxicity was encountered.

Isolation.—Early morning mid-stream specimens of urine were used, taken with the usual antiseptic toilet. A centrifuged deposit was examined microscopically for cells and organisms and cultured on McConkey's medium and on blood agar containing "dispersol L.N." to prevent swarming (Mackie and McCartney, 1948).

By this technique results could only be assessed in such terms as "heavy growth," "light growth," and so on. Nevertheless, there was clinical evidence of improvement in spite of failure to sterilize the urine and therefore approximate viable counts were undertaken in the later stages of the experiment (Group II). Again early morning mid-stream urines were used. These were refrigerated at 4° C. immediately after collection, and examined as rapidly as possible after removal from the refrigerator, in order to minimize bacterial multiplication. Tenfold serial dilutions of the urine samples were made in sterile physiological saline, down to 1 in 1 million. Of these dilutions a standard 4 mm. loopful was then plated on blood agar containing "dispersol L.N." Colonies were counted after overnight incubation at 37° C.

Identification.—Identification of *Proteus* was based on the usual cultural, morphological, and biochemical criteria (Wilson and Miles, 1946; Kauffmann, 1951; Cooke, 1948). Urease production was tested on Christensen's medium.

Sensitivity.—A tube dilution method in nutrient broth was tried at first, but was abandoned owing to difficulty in dissolving "furadantin" to produce a suitable stock solution.

A range of 2 ml. nutrient agar slopes in bijou bottles was therefore prepared by adding a stock suspension of "furadantin" (50 mg. per ml.) in sterile distilled water in amounts small enough not to interfere materially with the strength of the gel, the stock solution being diluted as required for the lower concentrations. By

trial and error it was found that 0.5, 1, and 5 mg. per ml. final concentrations of "furadantin" in nutrient agar covered the range of sensitivity of most strains of *Proteus* encountered. These were the concentrations used.

After preparation, the slopes were refrigerated for 24 hours to permit diffusion, and subsequently stored at refrigerator temperature if not required for immediate use.

In testing sensitivity of *Proteus* strains, the inoculum was one 4 mm. loopful of a five- to six-hour growth of the organism in digest broth, spread on the surface of the agar slope. Reading was done after 18 hours' incubation at 37° C. and was reported simply as "growth" or "no growth," visible with a hand lens or naked eye. No attempt was made to distinguish between bacteriostatic and bactericidal effects.

Results

Clinical.—In the *Proteus* infections studied in this series no characteristic syndrome emerged. Some infections were symptomless; in others, patients reported nocturnal frequency and scalding micturition, and remission in these during treatment was accepted as clinical evidence of improvement.

Results were assessed in 12 cases by careful questioning (Table I).

TABLE I
RESULTS IN 12 CASES

Antecedent Condition	Clinical Response	No. of Cases
Post-prostatectomy ..	Symptom-free at end of course	6
	Improved	1
	Unchanged	1
Post-prostatectomy-stricture or carcinoma of prostate	Improved (These four cases relapsed clinically after the end of the course of "furadantin")	4

Bacteriological.—From this point of view the cases fall into two groups:

Group I: No quantitative estimate of *Proteus* bacilluria made.

Group II: Quantitative estimate made.

Group I.—These results are not reported in detail, since little consistent change was detected during treatment.

Group II.—Detailed consideration of colony counts shows a falling off in numbers in the test group (Table II) compared with the controls (Table III). For brevity, results are shown as numbers of patients in whose urine colony counts of less than 30 per plate were seen. In most instances, those plates showing more than 30 colonies were quite uncountable, but most of those showing less than 30 showed none.

TABLE II
COLONY COUNTS IN TEST CASES

Dilution of Urine	Day of Treatment					After Treatment	
	1	2	3	4	5	6	7
1/10	1	1	1	2	1	1	1
1/100	1	1	1	2	1	1	1
1/1,000 ..	1	2	1	3	2	1	1
1/10,000 ..	1	2	2	3	3	2	1
1/100,000 ..	1	3	3	3	3	2	1
1/1,000,000 ..	1	3	3	3	3	2	1
Total examined	3	3	3	3	3	2	2

TABLE III
COLONY COUNTS IN CONTROL CASES

Dilution of Urine	Day of Observation				
	1	2	3	4	5
1/10	0	0	0	0	0
1/100	0	0	0	0	0
1/1,000	0	0	0	0	0
1/10,000	1	1	0	0	0
1/100,000	Not counted		0	1	0
1/1,000,000	—	Not counted	—	—	1
Total examined	2	2	2	2	2

Sensitivities.—These were expressed as "growth" or "no growth" at the concentrations tested. Fifty-three cultures of *Proteus* isolated from urine were tested in order to arrive at some sort of sensitivity base line. Of these, 44 were sensitive to 1 mg. per ml. and resistant to 0.5 mg. per ml.: six were sensitive to 0.5 mg. per ml. and resistant to 0.1 mg. per ml.: three were resistant to 5 mg. per ml.

Strains.—Forty-eight of the strains tested for sensitivity were typed biochemically, using reactions with mannite, maltose, gelatin, and sucrose, and their production of H_2S and indole.

Of these, seven were *Vulgaris*, two *Mirabilis*, one *Morganii*, eight *Rettgeri*, and the remainder atypical. The 30 atypical strains fell into groups, consistent within themselves, containing organisms not necessarily isolated from one patient only. It is hoped to investigate these biochemically atypical strains further at a later date.

No clear relation between biochemical type and sensitivity to "furadantin" was observed.

Discussion

The assessment of a method of treatment for *Proteus* bacilluria is complex. Clinical methods tend to be subjective, since no characteristic syndrome is seen, and, indeed, infections may be symptomless.

Bacteriological methods are more objective, though there are many problems. Special methods are essential to ensure that *Proteus* does not multiply excessively in the sample before it is tested in the laboratory. With experimental courses of treatment, if complete cure is not obtained, quantitative methods of assessment are necessary. Preliminary testing in our series has shown that more than one biochemical strain or variant of *Proteus* may be recovered from a single urine from a case of *Proteus* bacilluria, and the possibility that one of these strains is resistant to "furadantin" has not yet been explored.

With these reservations, it is considered that the test cases in Group II show a striking difference from the controls, though at present both are few.

It is possible that longer courses of treatment might give more clear-cut results.

It is hoped to provide serological confirmation for the isolation of a high proportion of biochemically atypical strains of *Proteus*. No attempt was made to identify *Providence* strains, since rapid urease production was accepted as a criterion of *Proteus* for the purpose of the experiments.

Furadantin appears to exert some beneficial effect on *Proteus* bacilluria, and merits further trial in this condition.

Summary

Twelve cases of *Proteus* bacilluria were treated with "furadantin" for five days. At the end of this period six patients were symptom-free, five were improved, and one was unchanged.

Eight of these cases were assessed bacteriologically, three of them by a rough quantitative method. Those tested quantitatively showed bacteriological evidence of improvement.

None of five control cases observed for periods of five days showed clinical or bacteriological evidence of clearing or remission of the infection.

Fifty-three cultures of *Proteus* isolated from urines were tested for sensitivity to "furadantin," and results are reported.

Forty-eight of these strains were typed biochemically. Of these 30 were biochemically atypical, but fell into several groups homogeneous within themselves.

We wish to express our indebtedness to Mr. H. K. Vernon, consultant surgeon, St. James's Hospital, Balham, for access to clinical material, and to Messrs. Duncan, Flockhart & Co. (Edinburgh and London) for supplies of "furadantin."

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A CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF THE THREE BILE PIGMENTS IN SERUM

BY

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In studying the aetiology of the different types of jaundice, much importance has been attached to the amount of "indirect" and "direct" bilirubin present in the blood. Cole, Lathe, and Billing (1954) have shown that in serum from patients with obstructive jaundice there are two bile pigments (pigment I and pigment II), which give a direct reaction in the van den Bergh test. Pigment I and pigment II can be separated from each other and from the indirect-reacting bile pigment, bilirubin, by reverse phase partition chromatography. Examination of the chromatograms has shown that there is considerable variation in the relative amounts of the three bile pigments. A quantitative method for their determination has therefore been developed so that a study of the clinical significance of the amounts of pigment I and pigment II can be made. The possibility that quicker and simpler methods for their determination might be employed has also been examined.

Methods

Apparatus.—It was not possible to elute the three bile pigments quantitatively from the usual type of partition chromatogram. A column (Fig. 1) was therefore designed so that, after separation of the three pigments, the supporting kieselguhr could be removed and divided into three portions, each of which contained a pigment. It was made from two 5-ml. syringes, the barrels of which were interchangeable. The hollow plungers were cut into three lengths (A, B, and C), the connecting surfaces were ground and a piece of glass tubing was fused to the top end (A). The nozzle of the syringe was removed from the barrel and annealed to portion C of the plunger. Portions A, B, and C were kept in position by the barrel. A length of fine-bore polythene tubing was fitted to the nozzle, which contained a small piece of cotton wool; this tubing was used to regulate the flow of solvents through the column by adjusting its height.

Solutions.—The following are required:—

(1) Saturated ammonium sulphate solution.

(2) Ethanol R.R.

(3) Solvent system: 50 ml. butanol; 45 ml. water; 5 ml. 0.05 M phosphate buffer, *p*H 6.0 (Cole, 1933).

(4) Freshly prepared alcoholic solution of diazotized sulphanilic acid (*p*H 4.1): 10 ml. 0.1% (w/v) sulphanilic acid in 0.25 N HCl; 0.3 ml. 0.5% (w/v) sodium nitrite; 20 ml. 1.43% (w/v) anhydrous disodium hydrogen phosphate; 90 ml. ethanol.

Procedure.—The chromatogram was prepared by stirring 1.5 g. silicone-treated kieselguhr (Howard and Martin, 1950) with 0.75 ml. of the upper phase of the butanol system and then gradually adding 10 ml. of the mobile aqueous phase until a smooth slurry had been made. The mixture was rapidly poured into the column and the excess fluid was drained off until the surface was just dry. The kieselguhr column held approximately 5 ml. mobile phase and was about 8.5 cm. long.

A protein-free solution of the pigments was obtained by adding to 1 ml. serum 0.18 ml. saturated ammonium sulphate solution and 2.5 ml. ethanol (Cole and Lathe, 1953). After standing in the dark for half an hour, the precipitate was removed by centrifugation and a sample of the supernatant solution, containing 50–100 μg. total bile pigments, was taken to dryness *in vacuo* at room temperature. If the serum contained large amounts of bilirubin (which is not soluble in the mobile phase) then a small amount of the silicone-treated kieselguhr was added just before drying was complete together with 1 ml. ethanol; the bilirubin became adsorbed on to the kieselguhr and the solution was taken to dryness. During the drying procedure care was taken to ensure that there were no losses of pigment due to the fluid creeping up the side of the tube. If there are several specimens for analysis and the serum has a low lipid content, then the drying may be conveniently carried out in a vacuum desiccator containing calcium chloride.

The dried supernatant was transferred quantitatively to the top of the kieselguhr column using a total of not more than 0.5 ml. aqueous phase of the butanol-water solvent system. The pigmented solution was run into the kieselguhr and, when the top was almost dry, 4.5 ml. aqueous solution was added. The column was kept out of the sunlight and draughts and the solution permitted to flow through at a rate of not more than 1 ml. every 3 minutes, until the top of the kieselguhr column was

again almost dry and separation of the pigments had been achieved as shown in Fig. 1. The plunger was then slowly pushed through the barrel so that the three portions containing the pigments could be separated.

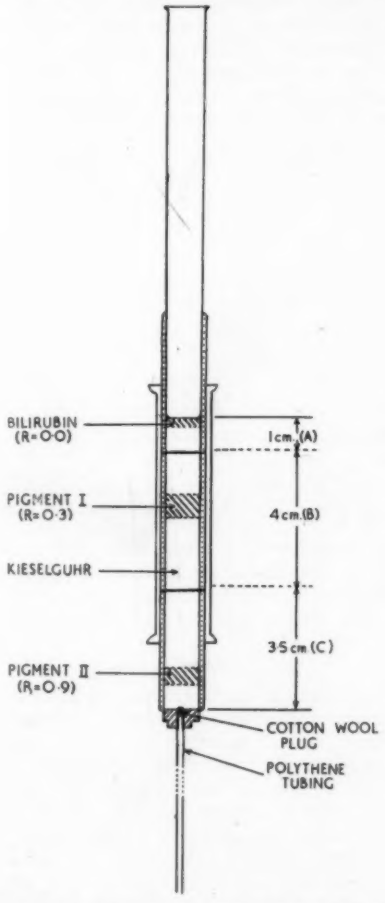

FIG. 1.—A microchromatographic apparatus made in three sections from the plungers of two 5-ml. syringes, which are kept in position by the syringe barrel. The three bands illustrate the relative positions of bilirubin, pigment I, and pigment II on the chromatogram.

The contents of each portion were transferred to glass-stoppered tubes. The pigments were converted to the corresponding azo-compound and extracted by shaking with 5–10 ml. alcoholic diazotized sulphanilic acid. After centrifugation the kieselguhr residue was re-extracted with 5 ml. ethanol. The solutions were combined for each pigment and the volumes measured. After 30 minutes readings were made in a Beckman spectrophotometer at 525 mμ and the proportions of bilirubin, pigment I, and pigment II calculated. A methyl red standard (King and Coxon, 1950) was used.

Determinations of serum bile pigments were made using the method of Malloy and Evelyn (1937), readings for the "direct" bilirubin being taken after 30 minutes; "1 minute bilirubin" was determined as described by Ducci and Watson (1945).

Experimental

Reproducibility of Results.—Table I shows the results of five analyses carried out on one specimen of serum whose total bile pigment content was 20.8 mg./100 ml. serum. The coefficients of variation for bilirubin, pigment I, and pigment II, were 4.7%, 5.2%, and 5.2%, respectively. No attempt has been made to study the reproducibility of results at different bile pigment levels.

Chromatographic and Malloy-Evelyn Methods for "Direct Bilirubin" Compared.—The method of Malloy and Evelyn (1937) has been widely used in clinical circles to determine the amount of "direct bilirubin" in the serum of jaundiced patients. The results obtained from 27 sera using this method have been compared with those obtained chromatographically by adding the values for pigment I and pigment II (Fig. 2). Student's t test showed that there was no significant difference between the results obtained by the two methods.

Ten determinations of "direct bilirubin" were made by the Malloy and Evelyn method on the

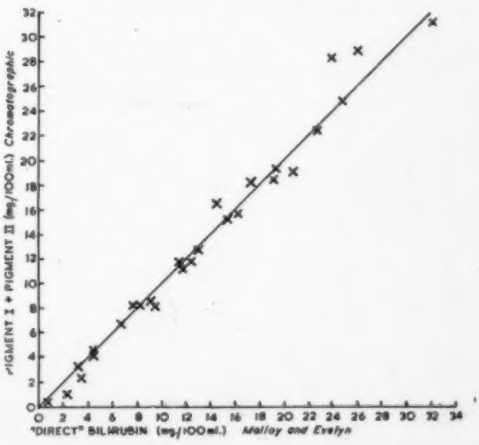
PIGMENT I + PIGMENT II (mg./100 ml.) Chromatographic
"DIRECT" BILIRUBIN (mg./100 ml.) Malloy and Evelyn
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34

FIG. 2.—A comparison of the results for "direct bilirubin" obtained by the Malloy and Evelyn method and the chromatographic method.

specimen of serum used in the study on the reproducibility of results (Table I). A mean value of 16.75±S.D. 0.41 mg. per 100 ml. serum was obtained. This gave a coefficient of variation of 2.4% compared with 2% for the chromatographic method.

TABLE I
RESULTS OF FIVE ANALYSES ON ONE SPECIMEN OF SERUM

Specimen	Serum Bile Pigments (mg./100 ml.)			
	Bilirubin	Pigment I	Pigment II	Total Direct Pigment
1	4·23	8·84	7·53	16·37
2	4·04	9·75	7·26	17·01
3	4·25	9·79	6·60	16·39
4	3·81	10·18	6·88	17·06
5	3·98	9·42	7·26	16·68
Mean	4·06	9·59	7·11	16·70
Standard deviation	0·19	0·50	0·37	0·33
Coefficient of variation	4·7%	5·2%	5·2%	2·0%

"1 Minute Bilirubin" and Pigment I.—Ducci and Watson (1945) have suggested that the diazotized bilirubin formed during the first minute of the direct diazo reaction in the Malloy and Evelyn method is due to the presence of a promptly reacting bilirubin, the remainder of the reaction being due to a delayed component. The possibility that this "1 minute bilirubin" could be identified with either pigment I or pigment II has been investigated in the hopes of providing a quick method for their determination. Table II shows a comparison of the results obtained for pigment I and "1 minute bilirubin" determinations made on 22 samples of serum. Some similarity in the results was obtained when the serum contained pigment I as the dominant bile pigment, but none was obtained when pigment II was present in larger amounts than pigment I. Fig. 3 indicates that the amount of "1 minute bilirubin" present was more closely related to the total amount of direct-reacting pigment than to a particular pigment, a finding that agrees with that of Klatskin and Drill (1950).

TABLE II
COMPARISON OF VALUES FOR PIGMENT I AND "1 MINUTE BILIRUBIN" IN 22 SAMPLES OF SERUM

Specimen No.	Pigment I (mg./100 ml. serum)	"1 Minute Bilirubin" (mg./100 ml. serum)	Pigment I − "1 Minute Bilirubin" / Pigment I × 100 (%)
1	0·3	0·4	−33
2*	1·1	3·5	−218
3*	1·6	3·5	−119
4*	2·5	5·1	−104
5*	2·8	6·4	−129
6*	3·3	6·6	−100
7*	4·8	7·8	−63
8	5·2	6·4	+23
9*	5·5	12·7	−131
10	5·7	5·0	+14
11	5·7	5·1	+11
12	7·3	7·3	0
13	8·5	8·8	−4
14	10·3	13·0	−26
15	10·3	8·6	+17
16	10·4	10·8	−4
17	11·0	7·6	+31
18	13·3	17·5	−32
19	13·8	11·4	+17
20	21·2	16·2	+24
21	21·4	15·7	+27
22	22·6	20·4	+10
Mean	8·57	9·08	
Standard deviation	6·44	5·74	

* Specimen contained more pigment II than pigment I.

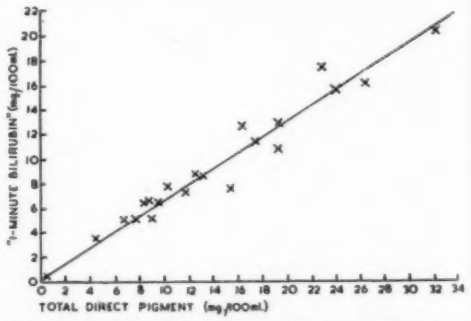

FIG. 3.—The relation between "direct bilirubin" and "1 minute bilirubin." Regression line $y=0.64x+0.24$ is superimposed.

Discussion

The instability of the direct-reacting bile pigments in serum is well known so that with any method for their quantitative determination considerable care must be taken to prevent their decomposition. The possibility of eluting both of these pigments from partition chromatograms was initially considered, but did not prove practicable since tailing of pigment I made the procedure too slow and elution of the pigment was not complete.

The use of small split columns enables the procedure to be carried out using about 1 ml. serum and has the advantage that the actual separation of the pigments takes less than 20 minutes. Provided excessive amounts of pigment are not used good separation can be achieved, and recoveries of 85 to 95% of the total pigment in the protein-free filtrate of the serum are obtained. Recovery experiments suggest that any losses that are incurred usually refer to the pigments as a whole and not to any particular pigment. The method is not easily applicable to sera whose total bile pigment concentration is less than 5 mg./100 ml. since

interference by lipids limits the amount of dried serum filtrate that can be run on a partition chromatogram.

The pigment adhering to an alcoholic precipitate of serum is known to be of the directly reacting type. The values given for bilirubin in serum, therefore, probably err on the high side. The accuracy of the chromatographic method is not greater than 5% so that it was agreeable to find that the values for the directly reacting pigments in serum by this method gave such good agreement with those determined by the method of Malloy and Evelyn, which involves no protein precipitation.

It was not possible to associate the prompt reacting "1 minute bilirubin" described by Ducci and Watson with either pigment I or pigment II, so that if information regarding the amounts of the two pigments is required the method described may be of use. If, however, only the total amount of direct-reacting pigments is wanted then the established and quicker method of Malloy and Evelyn will give equally reliable results.

Summary

"Bilirubin" as determined by the usual diazotization procedures consists of three related substances: bilirubin, and two pigments, which give a direct reaction in the van den Bergh test (pigment I and pigment II). These three bile pigments can be separated by reverse phase partition chromatography. A quantitative method for their determination is described.

The Malloy and Evelyn (1937) method for determining "direct bilirubin" in serum is shown to give a reliable estimate of the total amount of pigment I and pigment II present.

The promptly reacting "1 minute bilirubin" described by Ducci and Watson (1945) could not be identified with either of the directly reacting bile pigments in serum.

The author is indebted to the Medical Research Council for a personal grant and to Professor E. J. King for his advice and the hospitality of his department. She also wishes to thank Dr. G. H. Lathe for valuable discussions.

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THE THREE SERUM BILE PIGMENTS IN OBSTRUCTIVE JAUNDICE AND HEPATITIS

BY

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Many workers have observed that in obstructive jaundice and hepatitis hyperbilirubinaemia is due mainly to a rise in those pigments which give a " direct " reaction in the van den Bergh test. Cole, Lathe, and Billing (1954) have shown that there are two water-soluble pigments which give this reaction. They appear to be chemically related to bilirubin, but their exact structure is still unknown and they have been temporarily called pigment I and pigment II. Pigment II is the more polar of the two and is the principal pigment found in bile.

The metabolic relationship between the three pigments is not fully understood and it was hoped that a knowledge of the amounts of pigment I and pigment II in serum might give further information on this subject. Using the method described in the previous paper (Billing, 1955), the sera from 17 jaundiced patients have therefore been analysed for bilirubin, pigment I, and pigment II.

The clinical diagnosis of obstructive jaundice does not necessarily mean that the obstruction of the bile ducts is complete and there is also the possibility of some additional liver pathology being present. The effect of bile duct obstruction on bile pigment metabolism can be more easily studied if the obstruction is produced experimentally under standardized conditions. An analysis has therefore been made of the sera from a small series of rats, whose bile ducts were tied, and the results obtained will be compared with those from the pathological sera.

Methods and Materials

The bile pigment determinations were made as previously described (Billing, 1955). The human sera were obtained from 14 patients with obstructive jaundice of varied aetiology and three patients who had infective hepatitis. Obstructive jaundice was produced experimentally in seven rats by ligating the bile ducts under light anaesthesia (Weinbren, 1953). After periods of three, six, and 11 days the animals were sacrificed and bile pigment determinations were made on their sera.

Results

Obstructive Jaundice and Hepatitis.—The findings for the sera from 14 cases of obstructive jaundice and three cases of hepatitis are given in Table I. There was some variation in the proportions of the pigments, but in all instances the dominant pigment was pigment I, which accounted for 38 to 62% (mean 49%) total bile pigments. Bilirubin formed 18–39% (mean 25%) and pigment II formed 15–35% (mean 26%) total bile pigments. No association was found between the level of total bile pigments and the proportion of any one pigment in the serum although when the obstruction was of long standing the proportion of indirect bilirubin appeared to be greater.

TABLE I

BILE PIGMENTS IN OBSTRUCTIVE JAUNDICE AND HEPATITIS

No.	Clinical Diagnosis	Total Bile Pigments (mg./100 ml. Serum)	Bilirubin (mg./100 ml. Serum)	Pigment I (mg./100 ml. Serum)	Pigment II (mg./100 ml. Serum)
1	Obstructive jaundice	9	2·6	4·4	2·0
2	" "	11	2·6	6·1	2·3
3	" "	11	2·9	5·1	3·0
4	" "	12	3·0	4·6	4·4
5	" "	12	2·2	6·0	3·8
6	" "	15	3·2	8·5	3·3
7	" "	18	4·1	11·2	2·7
8	" "	21	4·0	9·8	7·2
9	" "	24	4·6	10·3	9·1
10	" "	26	7·5	10·4	8·1
11	" "	27	7·8	10·5	8·7
12	" "	30	11·7	13·8	4·5
13	" "	31	10·2	12·1	8·7
14	" "	39	13·7	19·1	6·2
15	Hepatitis ..	8	2·0	4·0	2·0
16	" 12/11/53	30	7·2	13·5	9·3
17	" 19/11/53	29	7·0	16·2	5·8
	" 4/12/53	39	13·7	18·3	7·0
	"	12	2·8	6·6	2·6

It was only possible to follow one case of hepatitis throughout the illness, and in this instance it was observed that when the total bile pigment concentration fell the proportion of bilirubin also fell. In

this small series no differences in the pattern of pigments given by sera from cases of hepatitis and obstructive jaundice could be distinguished.

Biliverdin was not detected in any specimens of fresh sera, although it was occasionally observed on the chromatograms of sera from cases of obstructive jaundice which had been left at room temperature for more than 24 hours.

Bile Duct Obstruction in Rats.—Unlike the sera from patients with obstructive jaundice, the chief pigment in the sera from rats with experimental obstruction of the bile ducts was pigment II (Table II) which accounted for 47 to 68% (mean 54%)

TABLE II
BILE PIGMENTS IN EXPERIMENTAL OBSTRUCTIVE JAUNDICE IN RATS

Days after Ligation of Bile Duct	Total Bile Pigments (mg./100 ml. Serum)	Bilirubin (mg./100 ml. Serum)	Pigment I (mg./100 ml. Serum)	Pigment II (mg./100 ml. Serum)
3	12	1·7	4·7	5·6
3	10	1·9	2·8	5·3
3	8	1·3	2·5	4·2
6	5	0·5	1·1	3·4
6	5	0·7	1·7	2·6
11	19	3·2	5·5	10·3
11	11	2·2	3·3	5·5

total bile pigments. Bilirubin was responsible for 10 to 20% (mean 16%) total bile pigments and pigment I for 22 to 39% (mean 30%) total bile pigments.

Discussion

There is some evidence to suggest that pigment I is an intermediate compound in the formation of pigment II from bilirubin since pigment I *in vitro* can be converted by gentle heating and evaporation to bilirubin and pigment II. Also, when diazotized in the van den Bergh test, bilirubin forms one azo compound (pigment A) and pigment II a different azo compound (compound B) while pigment I forms both pigments A and B (Billing, 1954).

If a rat, whose bile duct has been cannulated, is given an infusion of bilirubin, at a rate which exceeds the excretory capacity of the liver for bilirubin, then the direct reacting pigment which gradually appears in the serum is almost entirely pigment I while the pigment in the bile is pigment II (Weinbren and Billing, 1954). In newborn babies with haemolytic disease, directly reacting pigments are sometimes found in the blood in addition to bilirubin, and in several instances it has been noted that pigment I, without any pigment II, is present in amounts up to 60% of the total bile pigments. The conversion of bilirubin to pigment II for excretion in the bile, therefore, appears to take place in two separate stages.

In human bile more than 80% of the pigments present are found to be pigment II, and it was therefore anticipated that this would be the chief pigment responsible for the rise in serum bile pigments in obstructive jaundice. The results have shown that although this is so in the sera from rats with experimental obstructive jaundice, in sera from patients with obstructive jaundice and hepatitis the rise in the bile pigments is largely due to an increase in pigment I.

This finding might be explained on the basis of a species difference governing the renal threshold of the two pigments, but this is unlikely, since in human urine pigment I is usually present in greater amounts than pigment II. A more probable explanation is that in the diseased human liver, in addition to the obstruction preventing the excretion of the bile pigments, there is also some impairment in the conversion of pigment I to pigment II. A defect in the conversion of bilirubin to pigment I may also occur since bilirubin was present in larger amounts in human than in rat serum, and in long-standing cases of obstructive jaundice relatively greater proportions were present. The possibility that pigment II may be reconverted to pigment I or bilirubin by some organ in the body must be considered, but there is at present no evidence that such a conversion can occur.

Summary

The sera from 14 cases of obstructive jaundice and three cases of hepatitis have been analysed for bilirubin and the two pigments which give a direct reaction in the van den Bergh test (pigment I and pigment II). In all the sera the proportion of pigment I (38–62% total bile pigments) was greater than that of pigment II (15–35% total bile pigments) or bilirubin (18–39% total bile pigments).

A comparison of the results obtained from human sera and from rats with experimental obstructive jaundice has been made.

In obstructive jaundice, it is suggested that, in addition to the obstruction preventing the excretion of the pigments into the bile, there is also an impairment in the conversion of bilirubin to pigment I and of pigment I to pigment II.

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AN INVESTIGATION OF SERUM LIPIDS AND LIPOPROTEINS BY PAPER ELECTROPHORESIS

BY

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In view of the importance now attached to them it is remarkable that the lipoproteins of human serum received so little attention from early workers. In 1929 Machboeuf isolated an alpha-lipoprotein from horse serum and in 1935 McFarlane, in the course of ultracentrifuge studies of human serum, discovered the "X-Fraction" which was later shown by Pedersen to be beta-lipoprotein. The development of large-scale ethanol fractionation of human plasma pools led to a much better understanding of the lipoproteins, particularly beta-lipoprotein (Oncley, Gurd, and Melin, 1950). Recently there has been evidence that the lipids or lipoproteins are changed in some manner in atherosclerosis, and this has led to more extensive studies by fractionation and by ultracentrifugation. Paper electrophoresis provides an alternative and simpler method of investigation which needs only inexpensive apparatus and has the additional advantage of requiring a very small specimen of blood. After electrophoresis the strips can be cut lengthwise and stained by various methods to show the relative position of different components. Swahn (1952) and Kunkel and Slater (1952) have already reported the results of the electrophoresis of lipoproteins in a few sera; we have examined the lipid pattern in some 400 samples of serum and have distinguished from three to five components. An attempt has been made to correlate the patterns with the clinical condition and to identify some of the components on the paper.

Techniques

Electrophoresis.—The strips are normally run suspended over a bar in the apparatus described by Flynn and de Mayo (1951). The type of paper, and the amount of serum applied, depends on the particular investigation. For serum proteins we use Whatman No. 1 with 0.007 ml. of serum per inch; lipoprotein patterns that are to be scanned are run on Whatman No. 1 with 0.02 ml. of serum per inch; lipoprotein patterns for visual inspection are run on Whatman No. 100 or 3MM paper, with 0.04 ml. of serum per inch. For all strips barbiturate buffer, *p*H 8.6, ionic strength 0.05, is used. Direct comparison of the position of bands is never made between separate strips, as even adjacent strips may not be quite comparable. When protein-, lipid-, and cholesterol-staining bands are to be compared one wide strip is cut lengthwise after ruling clear marker lines to allow for exact positioning of the different pieces.

Staining Methods.—The following give good results:

Proteins.—The stain is 0.5% light green S.F. (Colour Index No. 669) dissolved in water containing 25% ethanol and 5% glacial acetic acid.

The strips are stained for 10 minutes, at room temperature, then washed in 2% acetic acid in tap water until the background is colourless.

Lipids.—The stain is a saturated solution of sudan black in 55% alcohol which has been matured for a few days and filtered before use.

The strips are stained for 30 minutes at room temperature, rinsed quickly in 50% alcohol, then washed out thoroughly in 40% alcohol. The background never becomes absolutely colourless, but in 40% alcohol very prolonged washing does not seem to reduce the intensity of colour in the actual lipid bands.

Cholesterol.—The Liebermann reaction is used, the amount of chloroform being greatly reduced to prevent the very soluble coloured product diffusing too rapidly.

Reagent.—Acetic anhydride, 8 ml., and 1 ml. chloroform are mixed, then 2 ml. concentrated sulphuric acid added drop-wise with constant cooling and mixing.

Method.—The reagent proved to be unsuitable for spraying because it takes up moisture from the atmosphere and is extremely unpleasant to handle. A layer of the reagent, approximately the size of the paper, is spread evenly in the bottom of a glass dish. The paper is then laid in it, and the lid put on while the blue or green colour develops; about five minutes gives maximum colour development. If the strip has to be handled it can be rapidly washed in ether and blotted, but moisture from the atmosphere soon causes the colour to fade. With practice it becomes easy to apply the right amount of reagent so that the paper is thoroughly wetted but there is no surplus. The reaction is given by both free and esterified cholesterol. The free cholesterol reacts instantaneously, but with the esters it is one or two minutes before the colour appears.

Other Staining Methods.—Iodine vapour, dichromate and haematein (Hack, 1953), and osmium tetroxide were used in an attempt to distinguish between cholesterol, fat, and phospholipid. It was found that both the iodine vapour and the dichromate and haematein stained the protein as intensely as the lipid, and that osmium tetroxide stained all lipids without discrimination.

Extraction of Strips with Organic Solvents.—The lipids can be differentiated to some extent by extraction with organic solvents after electrophoresis. Ethanol was found to be the most useful solvent giving satisfactory removal of neutral fat and cholesterol. After electrophoresis the strip is put into a large stoppered boiling tube almost filled with ethanol, and shaken gently for two, four, or six minutes. Four minutes appears to be the optimum time, the pattern not changing appreciably on longer washing. The strip is then stained together with the unextracted portion.

Extraction of Serum with Ether.—Serum, 1 ml., was extracted with three successive 1-ml. portions of ether, either frozen to −23° C. or at 0° C. The serum and ether were kept well mixed for 10 minutes, allowed to separate, and the ether pipetted off. The extraction was almost complete at both temperatures.

Storage of Serum.—The serum can be stored for about four days at 4° C. without the fat pattern changing. Freezing at −10° C. causes no change in the pattern, and several sera have been stored in this state for many months without deterioration.

Results

General Description of Lipid Patterns.—There are two black-staining areas which we have called the alpha-lipid and beta-lipid zones. The alpha-lipid zone is level with the albumin and alpha$_1$-globulin of the protein pattern, and consists of two or three overlapping bands. The beta-lipid zone extends from the line of application to a position between the alpha$_2$- and beta-globulin and usually contains three components which vary considerably in their relative intensities.

A detailed description of the individual components follows, starting at the line of application or origin, marked O in Fig. 1.

Beta-lipid Zone.—The lipid deposits at the origin and in the trail.

Normal serâ give a faintly staining area extending from the line of application up to the beta-lipoprotein band; at the back it is sharply defined by the boundary of the area wetted during application of the serum, but in front the staining merges into the beta-lipoprotein band. With lipaemic sera this area is stained more intensely than usual; in the alcohol-extracted strips it is practically unstained. These deposits contain little cholesterol or phospholipid, and probably consist largely of neutral fat.

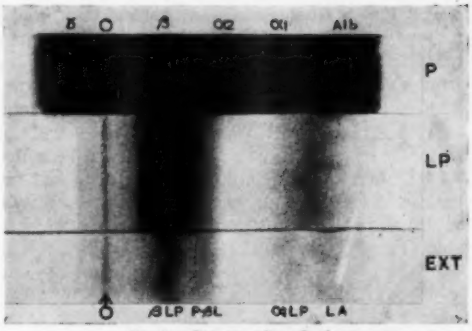

FIG. 1.—Coronary thrombosis.
P = protein, LP = lipoprotein, EXT = lipid in alcohol-extracted strip.

Beta-lipoprotein is an intense and sharply defined band level with the beta-globulin of the protein pattern which contains the bulk of the cholesterol and is also rich in phospholipid. It is greatly reduced by extraction with alcohol, leaving only a faint residual band.

With some sera the staining extends ahead of the beta-lipoprotein and appears to be due to an additional band which is partially obscured by the beta-lipoprotein. In the alcohol-extracted strips the beta-lipoprotein is greatly reduced, but this "pre-beta-lipid" is virtually unchanged and may appear as a distinct band. It is a prominent feature of the nephrotic pattern, and occurs frequently in coronary thrombosis.

Alpha-lipid Zone.—This consists of two or three overlapping bands which sometimes show slightly differing shades. The chief component, the alpha-lipoprotein, lies just behind the albumin and may be slightly brownish. In front of it level with the albumin is a slightly bluish band, which we ascribe to "lipalbumin." Sometimes there is another faint bluish band behind the alpha-lipoprotein. Alcohol removes most of the alpha-lipoprotein but leaves the lipalbumin so that the extracted strip shows a lipalbumin band and in some cases a faint band level with alpha$_1$-globulin (Figs. 1, 5).

Gamma-lipoprotein.—In a few cases pathological sera with a very high gamma-globulin level show a faint band of black staining level with the gamma-globulin which may be "gamma-lipoprotein."

Ether-extracted Serum.—Extraction of the serum with ether before electrophoresis makes no appreciable change in the protein pattern but removes almost all the lipid, although there is sometimes very faint staining in the alpha$_2$-globulin and albumin regions. Incomplete extraction may leave a deposit of lipid at the origin and in the trail.

Position of Cholesterol.—In normal sera, and in many with raised cholesterol, the Liebermann reaction shows the cholesterol of the beta-lipid zone to be limited to the narrow beta-lipoprotein band. However, in some cases of hyperlipaemia and of nephrosis with very high cholesterol and lipaemic sera there is in addition to the beta lipoprotein band a faint colour in the trail and pre-beta-lipid (Fig. 2). By using a double quantity of serum a trace of cholesterol can be detected in the alpha-lipoprotein.

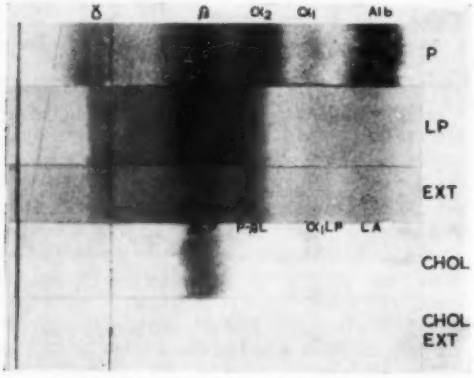

FIG. 2.—Nephrosis showing cholesterol distribution.

Position of Neutral Fat.—Four volunteers (one a diabetic) were given a fatty meal containing 80 g. of butter fat and the serum examined immediately before and three hours after taking the meal. In all cases the post-prandial serum showed some degree of lipaemia, and in three cases the black stain at the origin was considerably intensified.

Position of Phosphorus.—The sera of patients with polycythaemia were examined by electrophoresis 44–48 hours after receiving radioactive sodium phosphate (^{32}P), and the position of ^{32}P in the strip was determined by autoradiography (Fig. 3). The distribution of phosphorus closely resembled the distribution of sudan black staining. The highest concentration was level with the beta-lipoprotein band. The alpha-lipid bands, particularly the lipalbumin, were relatively rich in phosphorus and there were small concentrations in the trail and in the pre-beta-lipid. A band due to inorganic phosphate was present a considerable distance in front of the albumin. Alcohol extraction removed the bulk of the phosphorus from all components of the beta-lipid zone, but left that in the alpha-lipid zone almost unchanged.

Normal Serum Pattern.—The darkest part of the pattern (Fig. 4) is the beta-liproprotein band which

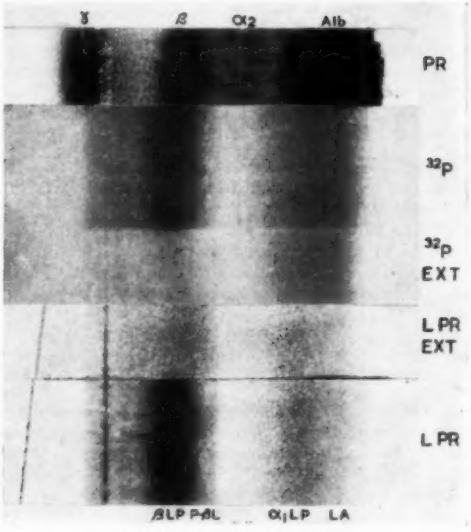

FIG. 3.—Phosphorus distribution.

is narrow and well defined; the alpha-lipid bands stain less darkly, but are considerably darker than the fat deposits at the origin or in the trail. The majority of normal sera have no pre-beta-lipid.

Nephrotic Syndrome.—Very characteristic changes in the serum protein and lipoprotein patterns occur in the nephrotic syndrome. The protein changes in the serum are well known; the albumin is always low, often very low, the alpha$_2$-globulin is much increased, and in severe cases gamma-globulin and beta-globulin are reduced. The urinary proteins tend to be complementary to the serum; appreciable concentrations of albumin, alpha$_1$, beta- and gamma-globulins are present but very little alpha$_2$-globulin.

The lipid pattern of the serum (Figs. 2, 5, and 6) shows dense staining throughout the beta-lipid

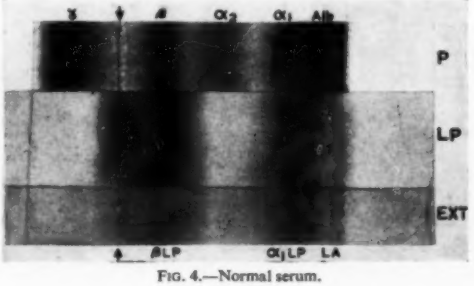

FIG. 4.—Normal serum.

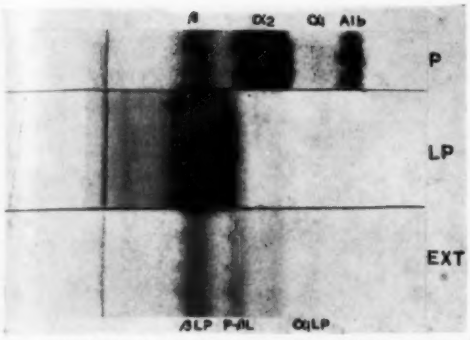

FIG. 5.—Nephrosis showing five bands. The original strip showed five bands, but two (α_1 lipoprotein and lipalbumin) do not show in reproduction.

zone, all components being increased. A characteristic feature is the dense and wide pre-beta-lipid band which invariably extends to the rear of the alpha$_2$-globulin and in extreme cases extends to its leading edge. In the alcohol-extracted strips the beta-lipoprotein band is greatly reduced leaving only a faint residual band, whereas the pre-beta-lipid is only slightly reduced in intensity. In a few cases with a very high alpha$_2$-globulin an additional faint band is seen corresponding in position with the alpha$_2$-globulin (Fig. 5).

The fat deposits at the origin and in the trail are also increased and vary with the degree of lipaemia. The cholesterol is increased but it is still largely confined to the beta-lipoprotein band except in very lipaemic sera where a small amount is found in the trail and pre-beta-lipid. In contrast all components of the alpha-lipid zone are diminished and in severe cases are undetectable. Extraction of the serum with ether removes practically all the lipid, but leaves the protein pattern virtually unchanged.

Diabetes.—The patterns found in diabetes are varied; some are normal, some show a moderate

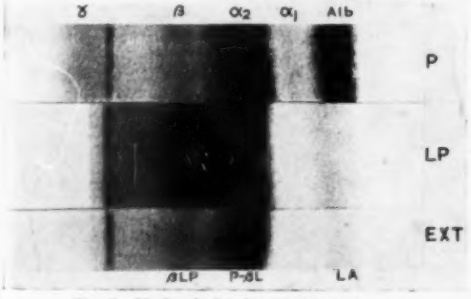

FIG. 6.—Nephrosis showing usual pattern.

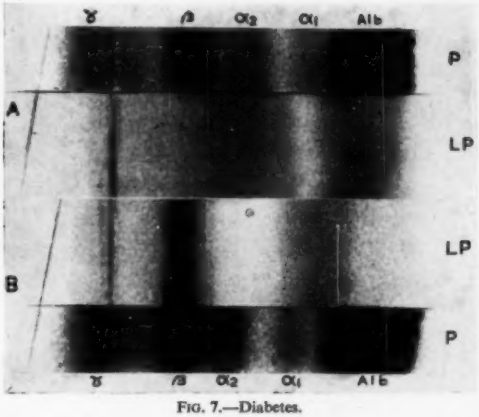

FIG. 7.—Diabetes.

pre-beta-lipid, and a few cases show a marked pre-beta-lipid extending to the front of the alpha$_2$-globulin. So far we have not found any clear correlation between the type of pattern and the clinical findings. In two cases of ketosis and coma the lipid front was swept forward, ahead of the alpha$_2$-globulin; on recovery the patterns returned to normal. Fig. 7A shows the serum of a patient with severe ketosis, almost in coma, and Fig. 7B his serum taken 20 hours later, when he had almost recovered.

Myxoedema.—The beta-lipoprotein band is denser than usual, as might be expected from the increased serum cholesterol, and is often very sharply defined. Usually there is no increase in the neutral fat deposits and the pre-beta-lipid band occurs only in a few cases (Fig. 8).

Xanthomatosis and Idiopathic Hyperlipaemia.—Two types of pattern are obtained in these conditions, one given by patients with hypercholesterolaemic xanthomatosis, and the other by patients with essential hyperlipaemia. In the former condition the serum is clear and the pattern obtained (Fig. 9A) is similar to that of myxoedema, with a very dense beta-lipoprotein band but no excess

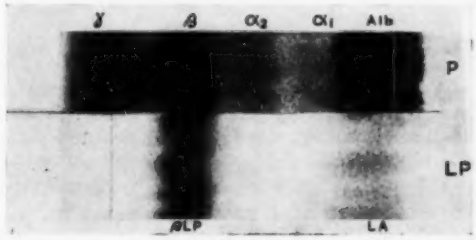

FIG. 8.—Myxoedema.

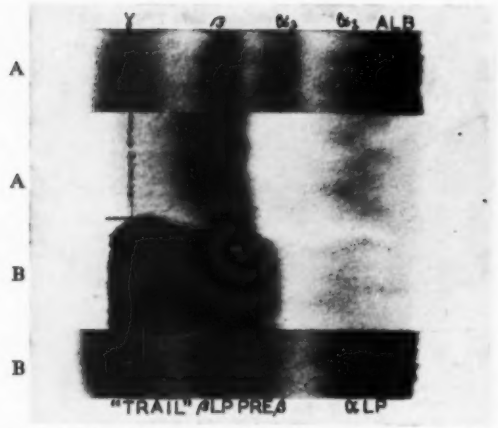

FIG. 9.—Xanthomatosis: A. Hypercholesterolaemia. B. Hyperlipaemia.

neutral fat or pre-beta-lipid. In the two cases of the latter condition we investigated the serum was very turbid, like milk, and gave a dense deposit extending from the origin to the alpha$_2$-globulin front (Fig. 9B). The beta-lipoprotein was not discernible as a separate band. In these cases the cholesterol was distributed throughout this zone although it was strongest in the beta-globulin position. One of these patients was treated first with intravenous heparin (9,000 units twice weekly) for 11 weeks, but this had little effect either on the serum lipid pattern or on the xanthomata. He was then put on a low-fat diet and after four weeks his condition began to improve, and the serum cholesterol to fall. Subsequently his intake of both fat and carbohydrate was restricted (he was still overweight) and further improvement occurred.

A comparison of the lipid analyses of the two sera shown in Figs. 9A and B is given below:—

	A. Hypercholesterolaemia	B. Hyperlipaemia
Total lipid extracted by alcohol-ether mixture (3:1) ..	2·0 g./100 ml.	4·2 g./100 ml.
Cholesterol	560 mg./100 ml.	580 mg./100 ml.
Phospholipid	310 „	550 „

On one occasion B's serum showed a total lipid of 5·5 g./100 ml.

We have found two cases of xanthomatosis which appeared to be a combination of these two conditions, with high cholesterol and moderately milky serum.

Jaundice and Cirrhosis.—In obstructive jaundice and in infective hepatitis both components of the alpha-lipid zone are reduced, and in severe cases

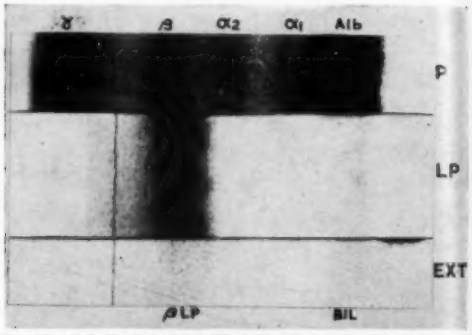

FIG. 10.—Infective hepatitis. The bilirubin strip is yellow and could not be reproduced in the photograph.

disappear altogether, while the beta-lipoprotein may be increased (Fig. 10). The serum lipid pattern of one patient was followed during the course of a severe and prolonged attack of infective hepatitis; the alpha-lipid bands disappeared during the severe stage of the disease, but returned during recovery. The main findings are summarized in Table I. The

TABLE I

MAIN FINDINGS IN JAUNDICE AND CIRRHOSIS

	Weeks after Onset of Jaundice	Serum Bilirubin (mg./100 ml.)	Lipalbumin and Alpha-lipoprotein Bands
Early phase ..	3 weeks	15	Both present
At height of illness	10 „	24	„ absent
During recovery	21 „	1·6	„ present
After „ ..	29 „	0·6	„ „

findings in cirrhosis are less consistent; the lipalbumin is frequently absent, but the alpha-lipoprotein is present in most cases (Fig. 11).

Coronary Thrombosis and Peripheral Arteriosclerosis.—An increased pre-beta-lipid has been found in a high proportion of cases of coronary thrombosis; a survey of these cases is now in progress and the preliminary findings suggest that the pre-beta-lipid may increase after infarction. The pre-beta-lipid is a less constant finding in peripheral arteriosclerosis.

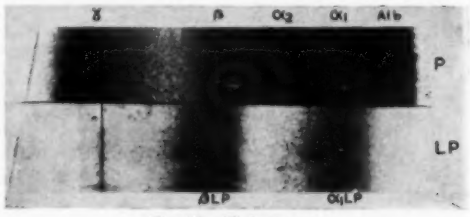

FIG. 11.—Cirrhosis.

Effect of Intravenous Heparin.—Injection of heparin produces a marked but transient change in the lipid pattern, but has little effect on the protein pattern (Dangerfield and Smith, 1955). In serum taken 20 minutes after injection the beta-lipoprotein is found to be accelerated into the alpha$_2$-globulin region (Fig. 12B) and the fat deposits in the trail are slightly diminished. The alpha-lipoprotein is displaced forward, apparently running ahead of the lipalbumin, and a corresponding band, staining very faintly for protein, appears ahead of the albumin but cannot be seen in the photograph. The first patients were given 15,000 or 12,000 international units, but in later cases it was found that 12,000 I.U. produced the same effects. With smaller doses the alpha-lipoprotein is not always accelerated. We have found this acceleration in as short a time as five minutes after injection, but our standard procedure was to take blood 20 minutes after injection; by 45 minutes the effect is less marked, and by two and a half hours the pattern has returned to normal.

Incubation of serum with 10 I.U. heparin per millilitre, which is approximately twice the concentration expected from the injected dose, does not change the lipid pattern. On the other hand, incubation with a small proportion (1 in 10) of plasma taken from patients 20 minutes after receiving intravenous heparin produced acceleration of the beta-lipid (cf., Comfort, 1953). This accelerating factor appears to be stable when the serum is stored for a few days at 4° C., or for some weeks frozen at – 10° C.

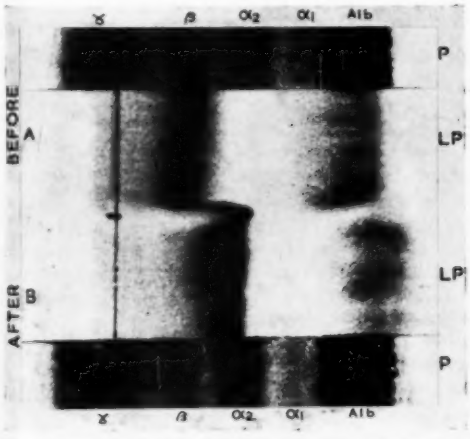

FIG. 12.—Heparin: A. Before intravenous heparin. B. After intravenous heparin.

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Discussion

In this paper we have described the patterns obtained when electrophoretic strips are stained with sudan black. For the sake of simplicity the black-staining components have been referred to as lipids or lipoproteins, but in fact we have not attempted to differentiate between free lipids, lipoprotein and lipo-polypeptide complexes, and substances not generally classed as lipids which have an affinity for sudan black. The alpha- and beta-lipoproteins are already well established in the literature; the other components we prefer to describe merely as lipids until more detailed information on their structure is available.

The affinity of various lipids for sudan black in 55% ethanol has been tested; olive oil, cholesterol oleate, and animal fats such as lard and mutton fat are intensely stained; cholesterol stearate and lecithin are moderately deeply stained, while free cholesterol and oleic acid give only a faint coloration. It is possible that non-lipid substances have a weak affinity for this stain and that high concentrations of protein, albumin in normal serum, gamma-globulin in cirrhosis and myelomatosis, or alpha$_2$-globulin in nephrosis give rise to faint bands. In this connexion it is particularly difficult to assess the significance of the lipalbumin band. A sample of human serum mercaptalbumin prepared from the five times recrystallized mercuric dimer was found to stain faintly with sudan black (cf., Swahn). On the other hand several sera with a normal or almost normal albumin concentration, and apparently no jaundice, showed no trace of lipalbumin, while certain body fluids and nephrotic urines with a large amount of protein showed no staining corresponding with the protein bands. The evidence is thus conflicting; if there is a lipid component associated with albumin, it appears to be very resistant to chemical separation, whereas if the protein itself is taking up the dye its affinity must be changed in certain pathological conditions. Until we have more evidence we propose using the term "lipalbumin" for the sudan-staining band corresponding with albumin, but do not wish to imply a true lipoprotein nature for it.

Comparison of serum taken before and after a fatty meal suggests that the lipids at the origin are probably neutral fat droplets deposited during application to the paper. The diffuse staining in the trail may be due to the deposition of lipid articles at different times during electrophoresis, or alternatively due to particles of various sizes migrating with a wide range of mobility.

The beta-lipoprotein has frequently been described in the literature, and occurs as a sharply defined

band in almost all the sera that we have examined. It is consistently the densest band, contains nearly all the cholesterol, has a high phospholipid content, and is largely extracted by alcohol.

The pre-beta-lipid is a less constant finding; it could be argued that it is the front of the neutral fat trail, and that the beta-lipoprotein is superimposed on the fat, dividing it into two parts. Against this view is the fact that the trail is almost completely extracted by ethanol, whereas the pre-beta-lipid is only partially extracted in some cases, and apparently not at all in others, particularly in nephrosis. The splitting of the alpha-lipid into two or three components is in agreement with the findings of Kunkel and Slater when they used a buffer of ionic strength 0.05. The separate bands are more clearly differentiated after alcohol extraction. In the unextracted strips the bands can be seen most clearly separated when all components are small; for instance, in cases of moderately severe nephrosis or liver disease. Sometimes only one alpha-lipid is discernible and this is usually the alpha-lipoprotein, but in three cases the lipalbumin was the only alpha component present.

The differential extraction of lipid from the strips by ethanol was unexpected, and we do not yet know what determines the solubility of different components. Virtually all the fat and cholesterol, and the bulk of the phospholipid, is removed from the beta-lipid zone, but only a small proportion of the phospholipid is removed from the alpha-lipid zone. The difference might be due to less soluble phospholipid components, to a different degree of unsaturation of the fatty acid esters, or to a firmer type of protein-lipid linkage.

Our findings on the distribution of cholesterol do not entirely agree with other reports in the literature. The amount of cholesterol found by various workers in alpha- and beta-lipoproteins separated by ethanol fractionation from human plasma pools is summarized by Oncley and Gurd (1953). All cholesterol recovered from Cohn's fractions I, II and III has been assigned to the beta-lipoprotein and that recovered from fractions IV, V, and VI to the alpha-lipoprotein. The figures for alpha-lipoprotein vary from 35 to 64 mg./100 ml., and for beta-lipoprotein from 132 to 193 mg./100 ml. with means of approximately 55 and 155 mg.%. Preliminary experiments in which we have cut out the fractions and extracted the cholesterol have given the same alpha/beta ratio of 1/3, a result which disagrees with that found when the Liebermann reaction is done directly on the paper. In one experiment three strips were run with 0.05, 0.1, and 0.2 ml. serum, respectively, and treated simultaneously with the cholesterol reagent. The coloration given by the alpha-lipoprotein from 0.2 ml. of serum was less than that given by the beta-lipoprotein from 0.05 ml. of serum, thus suggesting a ratio less than 1 : 4. This discrepancy may be due to the high proportion of protein in the alpha-region interfering with the reaction.

Radioactive phosphorus is distributed throughout the strip in proportion to the total lipid. This is in good agreement with the chemical determination of phospholipid by Kunkel and Slater, and suggests that it is an essential part of all lipid components, including the neutral fat droplets.

In all the pathological conditions investigated our results are in general agreement with the findings of earlier workers, both by electrophoresis (Swahn, 1952; Kunkel and Slater, 1952) and by chemical studies on separated plasma fractions (Barr, Russ, and Eder, 1953; Lever and Hurley, 1953). There are, however, a few minor differences; for instance, in nephrosis Kunkel and Slater found a large lipid peak in the alpha$_2$-globulin region but beta-lipoprotein was absent. We have examined more than 30 cases of nephrosis and find both beta-lipoprotein and pre-beta-lipid bands to be increased. The main variation is in the position of the pre-beta-lipid front.

The resolution of the lipids into more discrete components should eventually help in interpretation, and the appearance of a component characteristic of a pathological condition, in particular the pre-beta-lipid in nephrosis, should prove of more value than the measurement of the overall increase in fats in certain groups of fractions.

The three cases of hyperlipaemia that we have studied are remarkable in that the beta-lipoprotein is not much darker than the rest of the pattern, but is submerged in the heavy lipid staining, while the cholesterol, instead of being confined to the beta-lipoprotein, is distributed more or less uniformly throughout the beta-zone, with slightly stronger staining at the origin. Examination of the lipid patterns of patients with xanthomatosis will probably prove to be of considerable assistance in following the progress of individual cases, and may possibly help in elucidating the nature of the disease. It is tempting to speculate that deficient formation of beta-lipoprotein may be a fundamental feature of essential hyperlipaemia.

Much work has been done on the relationship between serum lipid and lipoprotein changes and the incidence of coronary thrombosis, atherosclerosis, and malignant hypertension; ultracentrifuge studies have been extensively pursued. The lipoproteins are of low density and "sediment" upwards if the

density of the serum is increased by the addition of strong salt solutions. Jones, Gofman, Lindgren, Lyon, Graham, Strisower, and Nichols (1951), working mainly at a density of 1.063, have found increased amounts of several lipoprotein fractions, particularly those they designate S_f 12–20 and S_f 30–100. Lewis and Page (1953), using a density of 1.21, have also found an increase in low-density components in malignant hypertension and in nephrosis, but they do not believe that it is justifiable to attribute atherosclerosis to any one of these components. They have studied some of their ultracentrifuge fractions in the Tiselius apparatus and have compared the ultracentrifuge and electrophoretic results. This correlation of ultracentrifuge and electrophoretic results is important and we are endeavouring to prepare the components that we have described in the ultracentrifuge.

In agreement with Lewis and Page we have not been able to demonstrate any change in the pattern that is really characteristic of the atheroma group of conditions; the pre-beta-lipid occurs more frequently than in normals, but it is by no means a constant finding, and it is probable that a quantitative method of assessment will be required to give any useful information. A combined ultracentrifuge and electrophoretic study may well be more profitable than either investigation alone.

In nephrosis the increased beta-lipids and decreased alpha-lipids is easily apparent and convincing; the difficulty here is to understand the significance of the effect. It is possible that for following the progress of individual cases the lipid pattern may be of greater value than the protein pattern, for it is our impression that the protein changes follow rather than precede the clinical changes.

These changes in the lipid pattern give an added interest to lipoproteins. In spite of the relative abundance of beta-lipoprotein in serum its origin, fate, and function are largely a matter of surmise. It is hoped that further work along the present lines may give some information either of theoretical or clinical value.

Summary

Methods for preparing and staining electrophoresis strips for protein, fat, and cholesterol are described, and the relative positions of these components in normal and pathological sera are shown.

The distribution of phospholipid has been determined by autoradiography of patterns from patients after treatment with ^{32}P.

A characteristic abnormal band, which is insoluble in absolute alcohol, is found in nephrosis. In many, but not all, cases of coronary thrombosis there is a similar band.

Intravenous heparin increases the mobility of the lipoproteins.

Photographs are shown of the lipoprotein patterns found in various conditions.

We wish to thank Dr. Iain MacDougall and Mr. J. S. Ajayi for assistance in the initial stages of this work, and Professor Blacklock for advice in preparing this paper.

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THE RELIABILITY OF THE DETERMINATION OF URINARY PROTEINS BY ELECTROPHORESIS ON FILTER PAPER

BY

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During the last few years several publications have appeared concerning the investigation of urinary proteins by electrophoresis on filter paper (Soulier, 1953; Slater and Kunkel, 1953; Fauvert and Hartmann, 1954). In these a comparative study was made on the basis of the electrophoretic diagrams between the different types of proteinuria and between the serum and urinary proteins in the various diseases accompanied by proteinuria.

The technical simplicity of the filter paper method permits the determination of urinary proteins on a large scale and makes the characterization suitable for clinical purposes.

Diagrams of urinary proteins often show weak protein bands even after sufficient concentration. Moreover, these diagrams often have to be made with a small amount of protein. In these cases, the various disturbing influences of the method may be of relatively greater influence.

Methods

Concentration of Urine.—The estimation of the urinary protein content is carried out using the sulphosalicylic acid and Esbach methods.

The urines are next concentrated by extracting their crystalloid solution by 15% aqueous carboxymethylcellulose (C.M.C.).* For this purpose small cellophane bags† are used, which contain about 40 ml. of urine. Depending upon the protein content, 20 to 40 ml. of urine is concentrated to 2 to 0.2 ml. If a high concentration is necessary, fresh C.M.C. may be applied. A 200-fold concentration can generally be achieved after four or five days. During the process of concentration the urines are kept in the refrigerator at 4° C.

Instead of C.M.C. a 25% aqueous solution of polyvinylpyrrolidone may be used, as was suggested by Slater and Kunkel and by Fauvert and Hartmann. The effectiveness of both solutions is about the same; polyvinylpyrrolidone, however, is much more expensive.

* A.K.U. Arnhem, Holland. Factory number XT 843.

† Dialysier schlang. Kalle & Co., Wiesbaden, Germany.

Electrophoresis.—The tank for electrophoresis described by de Wael (1953) was modified by us for a microtechnique giving good resolutions of the various protein bands within four hours.

In this tank, 36.5 by 43.7 by 37 cm. (Figs. 1*a* and 1*b*), two parallel glass rods (a) are placed 7 cm. above the bottom. The distance between the rods is 10 cm. Two small vessels (b), of which the inner walls (i) are just underneath the glass rods, contain the buffer (barbital buffer, *p*H 8.6; molar strength 0.1; 45.32 g. barbital sodium and 8.08 g. barbital dissolved in 4 l. of distilled water). Beside each vessel (b) is another vessel (c), which contains the electrodes (d) and, in addition, buffer. Both vessels (b) and (c) are linked by filter paper (f), damped with buffer. Thus a change of the *p*H of the buffer in vessel (b) will be prevented.

The filter paper used is Whatman 1. Strips, 24 by 4 cm. (h), are suspended over the two glass rods, the ends dipping in the vessels (b). The vertical distance between the surface of the buffer and the horizontal part of the strips must be at least 7 cm., otherwise too much buffer will be absorbed by the strips. Nine strips can be placed beside each other in the tank. So nine sera or urines can be investigated at a time.

After the strips are completely damped with buffer, 0.01 to 0.02 ml. of serum or 0.04 ml. of concentrated urine is brought on to the horizontal part of the strip in a narrow band parallel to the glass rods, at a distance of 1 cm. from the glass rod on the cathode side. According to the technique of van Os (1952) this is accomplished by means of a polished slide, a little shorter than the width of the strips, the material being evenly distributed along the margin. Fig. 2 shows how this slide is handled.

After the tank has been covered by a glass plate, the current is switched on and the voltage adjusted to 180 v. with the help of the two electrodes (g). In view of the shortness of the strips this tension difference is rather high, but the great advantage of this voltage is that within four hours a clear separation between the protein fractions will be obtained.

There appears to be much evaporation in the tank, which is shown by the many droplets of water on the walls. It is caused by the large volume of the tank

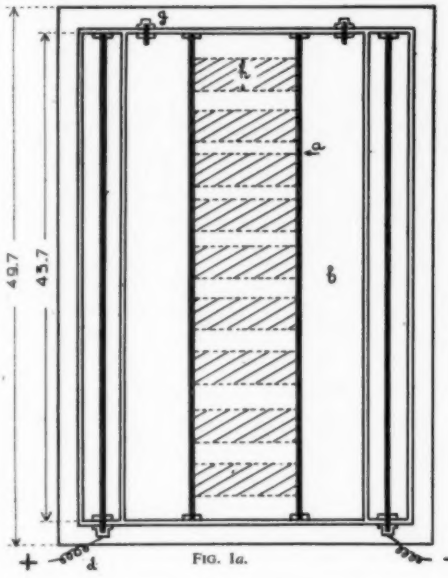

FIG. 1a.

FIG. 1b.

FIGS. 1a and 1b.—An upper and side view of the tank for electrophoresis (described by de Wael).

and the warming up of the strips by the high-voltage current (2.5° C. for four hours).

The high tension difference, the evaporation, and probably other factors as well appeared to contribute excellently to obtaining good resolution in very sharp bands. These sharp bands appeared to be of great assistance in the characterization of urinary proteins with very small amounts of proteins.

Fig. 3 is an example of two diagrams made from the urine and the serum of a patient with a nephrosis.

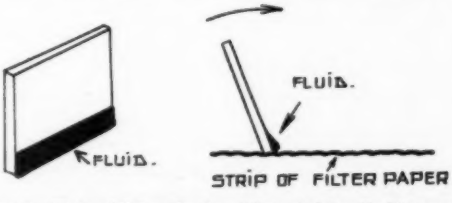

FIG. 2.—The mode of application of the material on the filter paper.

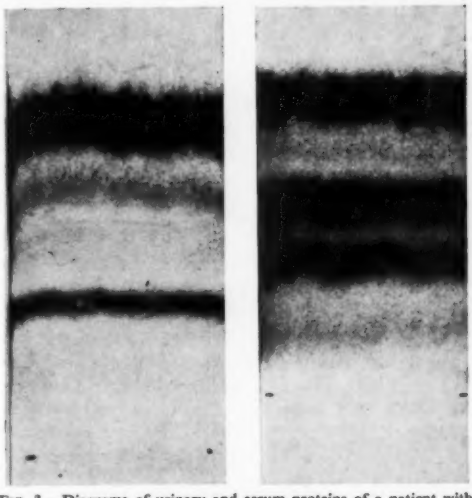

FIG. 3.—Diagrams of urinary and serum proteins of a patient with a nephrosis.

Staining.—After drying the strips for 15 minutes at 110° C., they are stained in a saturated solution of azocarmine B (Bayer) in methylalcohol, acetic acid, and water (5:1:4) for 15 minutes. The strips are next washed three times for five minutes in 10% acetic acid. After each washing fresh acetic acid is used. The diagrams are dried at room temperature.

The mutual ratio of the protein fractions is determined by cutting out the bands and by extracting them with 5 ml. 1 in 10 N. NaOH. The extinction of each solution is determined with the Beckmann spectrophotometer.*

The extinctions of the globulin proteins have to be corrected by the factor 1.5 in order to express the mutual ratio of the fractions to their nitrogen content.

TABLE I

FIRST STANDARD DEVIATION OF THE VARIOUS PROTEIN FRACTIONS IN DIFFERENT METHODS OF ESTIMATION

	Albumin	Alpha-globulins	Beta-globulins	Gamma-globulins
Electrophoresis in free solution (Dole, 1944)	2·8	0·7 1·3	1·9	2·5
Electrophoresis in free solution (Ewerbeck, 1950)	2·5	1·75	0·3	1·9
Filter paper electrophoresis (de Wael, 1953) ..	2·3	1·0	0·8	1·4
Present method of filter paper electrophoresis	2·4	1·0	1·4	1·48

Reproducibility of the Method for Serum

This was investigated by determining the standard deviation of the estimation errors for each fraction from 18 analyses of a serum sample performed on

* Franglen and Martin (*Biochem. J.*, 1954, **57**, 626) have shown the impossibility of obtaining absolute values for protein distribution by measurement of dye uptake on filter paper.—ED.

different days. Table I shows the results, together with those of the filter paper method, described by de Wael, and of the method of electrophoresis in free solution determined by Dole (1944) and Ewerbeck (1950). In comparison with these three methods, ours appeared to give even better results.

These standard deviations are only valid for the method described under strictly standardized conditions. Any change in the performance will immediately influence the results.

Quantitative Value of Diagrams of Urinary Proteins

For the analysis of serum 0.02 ml. of material is used, equalling approximately 1.4 mg. of protein. The minimal amount of protein, with which a suitable diagram is obtained, appeared to be about 0.2 mg. Because not more than 0.04 ml. of material may be applied to a strip, the urines must contain at least 0.5% of protein in order to give any results.

The influence of the variation in the total amount of protein upon the quantitative values of the fractions was investigated. A series of dilutions was made from a serum sample, and from each dilution a diagram with the same quantity of fluid. The mutual ratio of the fractions was determined from the different diagrams. Tables II and III show the results. Within a variation of 1.4 to 0.6 mg.

TABLE II
ELECTROPHORESIS ON FILTER PAPER WITH VARIOUS AMOUNTS OF PROTEIN FROM ONE SERUM SAMPLE

Total Protein (mg.)	Albumin %	Alpha-globulin %	Beta-globulin %	Gamma-globulin %
1·5	55	14	9	22
1·5	56	14	10	20
0·75	55	14	9	22
0·6	54	15	9	22
0·4	51	16	9	24
0·3	50	17	10	23
0·2	49	23	8	20
0·15	41	21	8	30

TABLE III
ELECTROPHORESIS ON FILTER PAPER WITH VARIOUS AMOUNTS OF PROTEIN FROM ONE SERUM SAMPLE

Total Protein (mg.)	Albumin %	Alpha-globulin %	Beta-globulin %	Gamma-globulin %
1·4	55	8	7	30
1·2	52	11	6	31
0·9	52	11	6	31
0·9	54	9	7	30
0·8	54	10	7	29
0·5	51	11	7	31
0·3	47	15	9	29
0·2	24	27	12	37

of protein no real change is obtained. However, by using less than 0.6 mg. a decrease of the albumin percentage is observed and appears to be progressive. The following factors are responsible.

Paper Blank.—The paper blank of the various bands can be measured by determining the amount of dye on a corresponding protein-free area of the same strip. Under our working conditions this paper blank showed almost no variation. The paper blank of each fraction is directly related to its surface on the diagram. In relation to the globulin fractions the albumins take only a small part of the diagram. The paper blank, therefore, will result in a smaller increase of the total extinction of the albumin band in comparison with the globulins and so in a relative decrease of the albumin percentage. This decrease appears to be less than 1% in the case of serum analysis with 1.4 mg. protein. With decreasing amounts of proteins, however, the constant paper blank causes an increasing error.

Albumin Trail.—Merklen and Masseyeff (1952) pointed out that a part of the albumin is absorbed during its migration over the filter paper. In order to measure the quantitative value of this "albumin trail" certain diagrams were made with various amounts of pure albumin.

In spite of the great variation in the amount of albumin the extinction of all trails shows approximately the same value. The absorption appears to be independent of the amount of migrating albumin. Apparently the absorption is a saturation phenomenon.

The quantity absorbed differs, however, with the type of filter paper used, as was suggested by Merklen

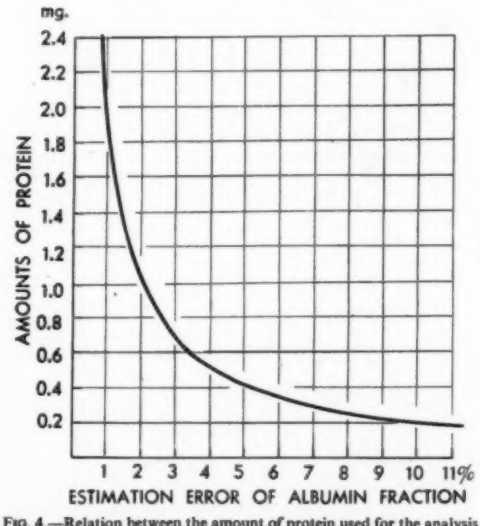

FIG. 4.—Relation between the amount of protein used for the analysis and the estimation error of the albumin fraction.

and Masseyeff. For Whatman 1 filter paper we found the absorption to be 0.7 μg. of albumin/cm.^{2} Under the same working conditions, described above, this quantity appeared to be for Nagel and Mockery filter paper 0.5 μg. According to Slater and Kunkel the globulins show a trail as well. We, however, could not detect one after saturating the filter paper with albumin.

The albumin trail causes a decrease of the albumin percentage and an increase of the fractions running behind the albumins. By using a normal quantity of serum proteins (0.02 ml.) this decrease appears to be about 1%. With smaller amounts of proteins, this constant albumin trail will cause a further fall in the albumin percentage.

Summary and Conclusions

Both the paper blank and albumin trail are sources of errors always made when serum proteins are investigated by means of filter paper electrophoresis. Using a normal quantity of serum (about 1.4 mg. in 0.02 ml.) these errors cause under our working conditions a seeming fall of the albumin percentage of 1 to 2.

With diminishing amounts of protein a further gradual fall in the albumin percentage will be observed. Fig. 4 shows the relation between the quantities of protein used for the analyses and the estimation error made for the albumin fraction. Within the range of 1.4 to 0.6 mg. the increase of the error remains just within one standard deviation of the albumin fraction, so only by using less than 0.6 mg. of protein will these errors result in an apparent fall of the albumin percentage.

Because not more than 0.04 ml. of material may be used for an analysis, the urines to be investigated must contain at least 1.5% of protein. With the method of concentration described, this protein content will easily be obtained in all cases of clinical proteinuria.

The minimal concentration of urines mentioned is only valid for the method described in this paper. By using another method, probably another value will be necessary. In any case the same kinds of errors will be made.

The method of filter paper electrophoresis of urinary proteins appeared to be simple and accurate, suitable for clinical purposes.

About 400 urines were investigated. The results will be published in another paper.

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AN APPARATUS FOR PAPER ELECTROPHORESIS

BY

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The apparent simplicity of filter paper electrophoresis has led to its use in the analysis of a wide range of mixtures of clinical and biological importance (see review by Martin and Franglen, 1954). The great interest in this technique has resulted in the publication of numerous papers on the design and construction of paper electrophoresis cells. During the past five years the writers have gathered practical experience of many of these instruments, and of the quality and reproducibility of the separations that can be obtained with them. As a result, a cell has been designed for high-quality electrophoretic separation of mixtures on filter paper; this apparatus is described in this paper. This cell has been in use for more than a year, and close on a thousand different analyses have been carried out successfully on it, reproducibility being of a consistently high order. The prototype was demonstrated at a meeting of the Society of Chemical Industry in 1954 (Franglen and Martin, 1954).

In designing such an apparatus, the following three requirements were considered to be essential: (1) Stable electrical conditions; (2) stable conditions in and about the paper on which electrophoresis takes place ("working strip"); (3) a means of applying the material to be analysed to the working strip with the minimum disturbance of conditions in the cell.

To satisfy the first requirement, it was found that, while it was easy to deliver a constant voltage by suitable stabilizers to all the electrophoretic cells tested, steady electrical conditions could not be maintained across the working strip when simple carbon electrodes were used. This is due to the fact that these electrodes readily polarize under the conditions normally used for paper electrophoresis. Moreover, in many buffers, carbon electrodes were found to disintegrate. Reversible silver-silver chloride-potassium chloride electrodes were used in the apparatus described here, and were found to be perfectly satisfactory. The surface area of each electrode is about 60 cm.^{2} when silver wire of 0.1 cm. diameter is used; this area is ample to deal with any current normally required. Moreover, the electrode carriers are designed so that other ductile metals, such as copper or platinum, can be used.

To satisfy the second requirement, it is necessary to ensure that (1) the buffer solution is evenly distributed over the whole working area of the paper; (2) the working strip is maintained at a controlled even tension; (3) changes of temperature and of water vapour content of the atmosphere about the working strip are reduced to a minimum. As pointed out by Durrum (1950), the inverted V electrophoresis apparatus cannot maintain an even distribution of buffer solution over the working strip. At the same time, the type of cell which involves continuous or intermittent support of a horizontal working strip must introduce side-effects difficult to control and to compute although the horizontal position of the working strip does result in even distribution of buffer solution over the working area. It was decided, therefore, to use a cell in which the paper was horizontal, but supported only at the ends where electrophoretic separation should not take place. In designing such an apparatus, it was essential to eliminate any sag that might occur in the working strip as it became saturated with buffer solution. This has been achieved by stretching the paper between two glass rods, the position of which can be adjusted to take up any sag and to maintain an even tension in the paper. Just above and below the working strip are two surfaces from which continuous evaporation of buffer solution may take place. This rapidly and completely saturates the atmosphere immediately around the working strip with water vapour, and results in the maintenance of a steady temperature in the apparatus. Measurements with thermocouples set in the working strip showed that the rise in temperature in an overnight run was no more than 0.30° C., even when potential differences as great as 10 V./cm. were applied to the paper. The apparatus takes a working strip 20 cm. in width on which six or more analyses can be carried

out simultaneously. The analysis is normally carried out in air, but, when necessary, it may be conducted in an atmosphere of nitrogen or any other gas.

To satisfy the third requirement, the material to be analysed is laid on the paper through a small entrance port in the lid of the apparatus. The comparative smallness of this opening together with the use of equilibrating devices within the apparatus result in very little disturbance of internal conditions of the cell during the application. After the material is laid on, the port is sealed by an air-tight lid.

Technical Section

General Description of Apparatus.—A general view of the apparatus is shown in Fig. 1, and details of internal fittings are shown in Fig. 2. In its present form, the apparatus is a rectangular box measuring 28 × 30 × 8 cm. made of thick perspex. To ensure complete air-tightness, a recessed rubber washer of square section 0.6 cm. thick (A, Figs. 1 and 2) is set into the rim of the box, and on to this a perspex lid is clamped by hinged bolts (B, Fig. 2). Electrode vessels (C, Figs. 1 and 2) are cemented on to the outside of the main box, and are connected through the main walls to the buffer chambers by six channels (D, Figs. 1 and 2). These channels are rectangular in cross section and are of inverted U-pattern. Three pairs of buffer chambers (E_{1-3}, Fig. 2) lie within the main body of the box. The first outer and smaller pair (E_1, Fig. 2) are connected to the electrode vessels through the U-channels, and to the second inner pair of buffer chambers (E_2, Fig. 2) through nine small holes in the intervening walls (F, Fig. 2). These two pairs of buffer chambers have a total capacity of 600 ml. Hydrostatic equilibrium of the buffer solution in these chambers is secured by means of a small syphon set on the bottom of the apparatus connecting the inner pair of chambers. This syphon acts as an electrical shunt during electrophoresis, but the proportion of current passing through it is small, measurable and constant for any one buffer. The third pair of buffer chambers (E_3, Fig. 2) lie at right angles to the first and second, and have no connexion with either of them.

Fitting and Control of Working Strip.—The working strip (G, Fig. 1), a sheet of filter-paper of requisite texture measuring 20 × 32 cm., is held at each end by simple perspex clamps (H, Fig. 1). These clamps are kept in position in the buffer chambers E_2 by spring clips of

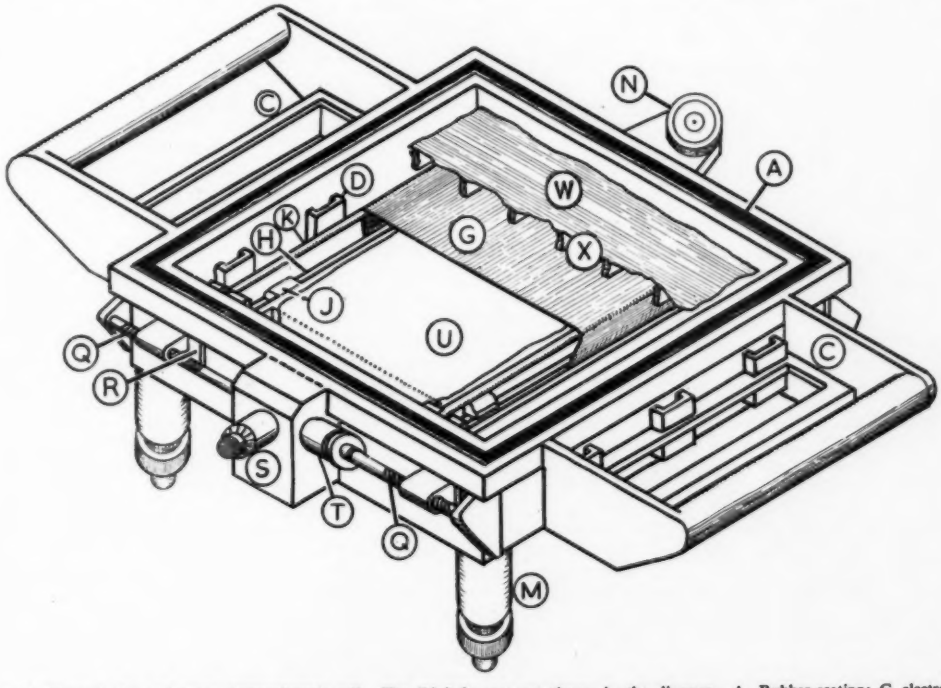

FIG. 1.—General view of paper electrophoresis cell. The lid bolts are not shown in the diagram. A, Rubber seating; C, electrode vessels; D, agar-potassium chloride bridges; G, working strip; H, working strip clamp; J, retaining tongue; K, glass rod; M, levelling legs; N, spirit level; Q, gear chain; R, spring-loaded plate; S, gear chain control knob; T, gear chain clutch; U, lower equilibrating paper; W, upper equilibrating paper; X, grid.

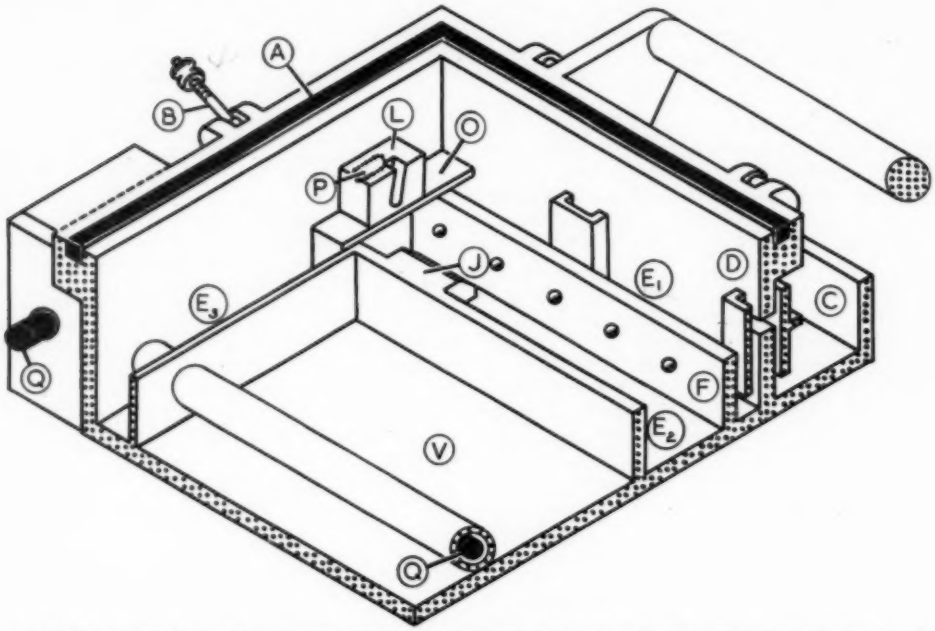

FIG. 2.—Details of internal fittings of paper electrophoresis cell. A, Rubber seating; B, lid bolts; C, electrode vessels; D, agar-potassium chloride bridges; E_{1-3}, buffer chambers; F, buffer chamber wall; J, retaining tongue; L, sliding block; O, sliding block shelf; P, slot; Q, gear chain; V, central well.

perspex under small projecting tongues (J, Figs. 1 and 2). The paper thus fixed passes up to and is stretched horizontally across two glass rods (K, Fig. 1) which rest in sloping slots cut in perspex blocks (L, Fig. 2). The whole apparatus is mounted on three levelling legs (M, Fig. 1), similar to those of an analytical balance, and is provided with a spirit level (N, Fig. 1) so that the level of the paper stretched between these rods can be adjusted to be exactly horizontal. The blocks, which support the rods, rest against the side of the apparatus on little shelves (O, Fig. 2) above the buffer chambers, and are connected through narrow slots (P, Fig. 2) to a gear chain (Q, Figs. 1 and 2) on the outside. The slots are made air-tight by spring-loaded plates (R, Fig. 1). On rotation of a centrally placed control knob (S, Fig. 1), the gear chain produces outward parallel movement of the glass rods to take up slack in the working strip; in cases where the sag is uneven, correction is made by altering the set of the glass rods to each other by means of a clutch (T, Fig. 1) on one side of the gear chain. The control knob is graduated in terms of distance between the two glass rods; this enables reproducible tension to be applied to the working strip, given the length and type of filter paper used.

Maintenance of Internal Atmosphere.—Two sheets of thick filter paper ("equilibrating papers") dip into the third pair of buffer chambers E_3, and run across the apparatus 1 cm. above and below the working strip. The lower paper (U, Fig. 1) is supported on a perspex plate covering the central well (V, Fig. 2); the upper paper (W, Fig. 1) rests on a light perspex grid (X, Fig. 1) over the working strip. Both sheets are thoroughly soaked in the buffer solution used on the working strip, and are kept wet by more buffer solution in the chambers E_3. By this means, the atmosphere about the working strip is kept completely saturated with water vapour, and any evaporation from the working strip is compensated by back-evaporation from the equilibrating papers. It must be noted that it is essential to use exactly the same buffer solution on the equilibrating papers and on the working strip; in practically all cases the use of water or other salt solutions on the equilibrating strips results in an unstable internal atmosphere, and in marked distortion of the electrophoretogram. When using buffers at *p*H above 9, no carbon dioxide should be present in the atmosphere if the buffer is to maintain its *p*H effectively. Using such buffers for electrophoresis, it has been found an advantage to be able to conduct the separation in a carbon-dioxide-free atmosphere. Accordingly, special inlets have been constructed in the lid to enable the apparatus to be flushed free of air, and filled with pure nitrogen or with other gases before electrophoresis is started.

Application of Material.—A narrow rectangular entrance port is cut in the main lid. The material to be analysed is applied through this port on to the wet

working strip by an "Agla" micrometer syringe (Trevan, 1925) or by other suitable means. A small slit, cut in the upper equilibrating paper directly under the port, allows access to the working strip with the minimum disturbance of the internal atmosphere of the apparatus. After the material has been applied, the port is sealed by a small rubber-seated lid clamped on by swing bolts. A number of lids with different entrance ports have been constructed to fit the main apparatus for use in various types of analyses.

Electrode System.—Direct current from a suitable stabilizer is supplied to the apparatus through the fully reversible silver - silver chloride - potassium chloride electrodes which are connected to the buffer chambers E_1 by agar-potassium chloride bridges. The electrodes are enclosed in protective carriers (Fig. 3) which fit snugly into the electrode vessels. These carriers are five-sided rectangular boxes of perspex, each holding a spirally grooved perspex rod of 1 cm. diameter. Fine silver wire, 0.1 cm. in diameter, cleaned of all traces of grease, is wound on to the groove by rotating the rod in the carrier, and is attached to the terminal on the upper side of the carrier. About 210 cm. of wire is used in each electrode giving an electrode area of over 60 cm.^{2} After being wound on to the rod, the wire is cleaned still further by being soaked for one minute in nitric acid (reagent grade) diluted to one third with distilled water, and followed by being rinsed thoroughly in distilled water. The electrode is stood in a plating bath of N/10 hydrochloric acid (reagent grade) so that the silver wire on the rod is completely immersed; the terminal head should not be below the surface of the liquid. The positive pole of a D.C. supply is connected to the electrode, while the negative pole is connected to a platinum electrode standing in the plating bath. A potential difference of 2 to 4 V. is maintained across the electrodes in the bath for not less than four hours, and preferably overnight. As plating proceeds, the current falls to a small value, and a dark pink deposit of silver chloride is formed on the wire. After plating the electrodes are rinsed free of hydrochloric acid with distilled water.

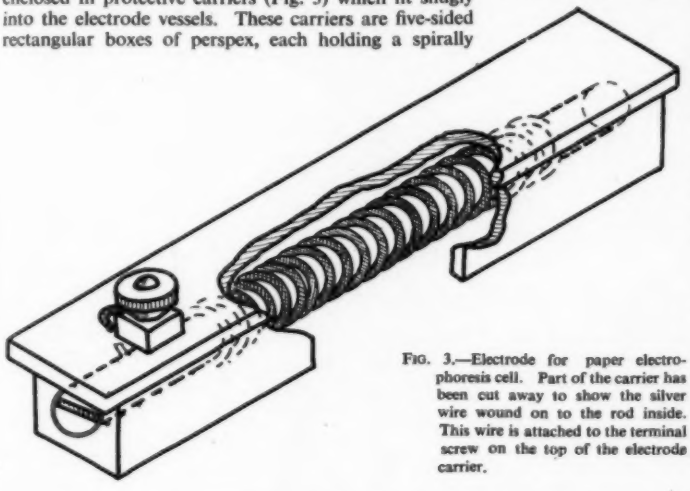

FIG. 3.—Electrode for paper electrophoresis cell. Part of the carrier has been cut away to show the silver wire wound on to the rod inside. This wire is attached to the terminal screw on the top of the electrode carrier.

Agar-potassium chloride bridges are formed between the electrode vessels and the buffer chambers E_1 by filling the U-channels described above with a hot solution of 1% (w/v) pure agar in 3.5 M aqueous potassium chloride (reagent grade). When this has set, the open tops of these channels are sealed with a layer of hard paraffin wax (Fig. 4). Provided they are prevented from drying out, these bridges can be used for months at a time without interference. When changing them, the paraffin seal is broken off, and the bridge channels are cleaned of agar with a fine test-tube brush. The electrode vessels are filled with 150 ml. of 3.5 M aqueous potassium chloride solution; the electrodes in their carriers are inserted into them and attached to a suitable D.C. supply. After each run, the polarity of the electrodes must be reversed to prevent deplating the negative side. They require no further attention other than an occasional replating in N/10 hydrochloric acid. When not in use, they must always be kept in distilled water or in 3.5 M aqueous potassium chloride solution, as they are ruined if allowed to dry out.

Method of Operation.—The apparatus is placed on the laboratory bench out of draughts, and trued by means of the attached spirit level. Alternatively it can be placed in a thermostatically controlled cabinet, if analysis at temperatures other than normal room temperatures is contemplated. The electrodes are set up as described above, and are connected to a stabilized D.C. supply. On the sheet of perspex, which covers the central well (V, Fig. 2), a thick sheet of filter paper (Whatman's No. 3MM) is laid and soaked with the buffer solution to be used in the analysis; this forms the lower equilibrating paper. More buffer solution, about 10 ml., is added to each of the chambers E_3. The dry working strip is mounted in the clamps, and is put in position in the apparatus; the clamps are fixed securely in the chambers E_2 by the perspex clips. The position of the rods is adjusted by the control knob until the paper is stretched taut. Buffer solution, 300 ml., is added to the chambers E_1 and E_3 on each side of the apparatus. The working strip is allowed to take up the buffer naturally; it should not be sprayed with buffer solution in an effort to hasten the uptake, as this often results in an uneven surface. When the working strip is completely wet the position of the rods is adjusted again to take up any sag and to give the required tension on the paper. The upper equilibrating paper, cut from a sheet of thick filter paper, is fitted on to the perspex grid; a narrow slot is cut in this paper under the entrance port in the main lid. Soaked in

buffer solution and resting on its grid, the upper paper is put in position in the apparatus, and its ends are tucked into the chambers E_2. The apparatus is sealed by clamping on the main lid, and is brought to equilibrium by passing the current at the voltage required for electrophoresis for at least one hour. After switching off the current, the entrance port is opened, and the material for analysis is applied to the paper. The apparatus is sealed and the current is switched on again for the period necessary for analysis. At the end of this time, the paper is removed from the apparatus, and detection of the components on it is carried out by the usual methods.

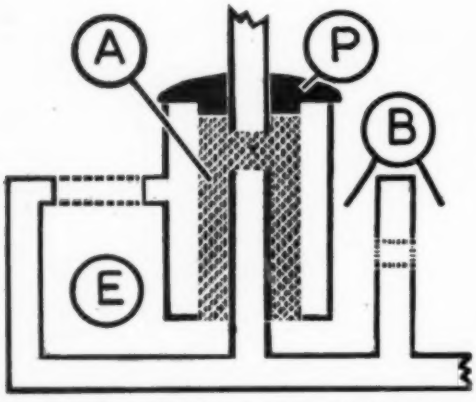

FIG. 4.—Vertical section of agar-potassium chloride bridges. A, Agar-potassium chloride jelly; B, buffer chambers; E, electrode vessel; P, paraffin wax seal.

Results

Fig. 5 (1, A, B and C) illustrates the analysis of protein components of normal and abnormal sera and plasma. These analyses were carried out on Whatman's No. 4 filter paper in 0.12 M diethylbarbiturate buffer of *p*H 8.6 at a potential difference of 3.5 V./cm. applied for 16 hours. We regard the accurate resolution of the α- and β-globulins as a sterner test of performance than the analyses of myelomatosis sera so frequently illustrated in the literature.

The two other analyses shown in Fig. 5 (2 and 3) illustrate artefacts which have nothing to do with inherent peculiarities of the proteins being analysed. The reproducibility achieved with the apparatus enabled these artefacts to be examined under controlled conditions.

Fig. 5 (2) illustrates a break in the qualitative texture of the paper matrix. Several sheets of the batch were shown to be defective.

Inadequate sealing of paper electrophoretic cells may lead to irregular migration of the components being analysed. The illustration in Fig. 5 (3) shows the appearance of a band of protein migrating past a deliberately produced air inlet, 0.5 cm. in diameter, open for six hours during analysis.

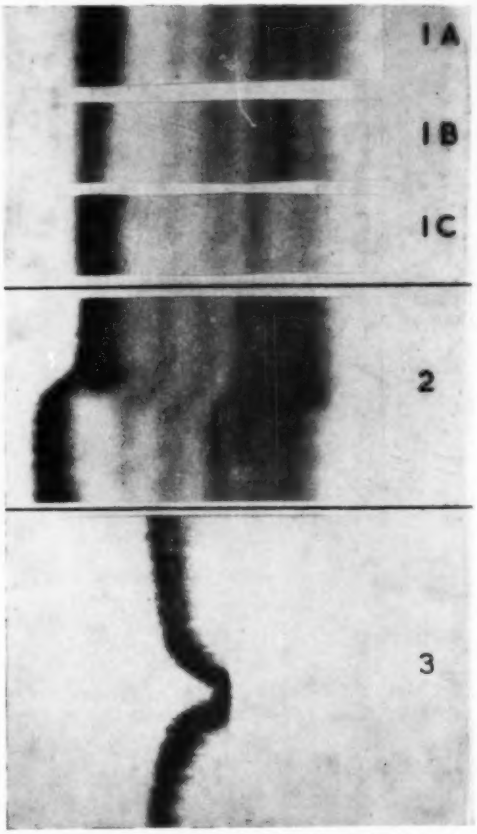

FIG. 5.—Examples of electrophoretograms run in the apparatus. (1A) Cirrhotic plasma; (1B) eczema plasma; (1C) normal serum; (2) cirrhotic serum run on faulty paper; (3) albumin run past a small air leak above the working strip.

Summary

An apparatus is described for the electrophoresis of material on filter paper. Although this apparatus is more complex than many hitherto described, experience suggests that the innovations produce a constancy and delicacy of performance in the hands of persons of such widely varying technical skill as to offset the initial expense the complexity must entail.

Without such consistent performance, it is not easy to compare the results of one laboratory with another, nor is it possible to distinguish certain irregularities, which are the result of artefacts, from those which are the result of true variations in the material analysed.

Our thanks are due to the Endowment Fund of St. George's Hospital for financial assistance during the work described in this paper.

The manufacture of an apparatus embodying the principles outlined in this paper has been undertaken by Messrs. Joyce, Loebl and Co., Ltd., Vine Lane, Newcastle-upon-Tyne, 1.

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J. clin. Path. (1955), 8, 150

THE USE OF BORATE BUFFER IN PAPER ELECTROPHORESIS OF SERUM

BY

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Electrophoresis of sera on filter paper is usually carried out in barbitone buffer. There are few references to the use of borate buffer, and, since we have observed that in this buffer the β-globulin components are better differentiated, it is considered of interest to record some of our observations.

Methods

Conditions of Electrophoresis.—The apparatus used was similar to that described by Franglen (1953). It consisted of a flat shallow "perspex" box in which a sheet of Whatman No. 1 paper was stretched horizontally with the ends dipping into the electrode solutions. The paper was first wetted with electrolyte, blotted, and placed in position in the apparatus. Serum (10 μl.) was then applied from a micropipette along a length of about 2.7 cm. of the starting line, which was placed towards the cathode end. The voltage applied was 14–15 V/cm. for two and a half to three hours and average current varied with the nature of the buffer used but in most experiments was 0.4–0.5 mA/cm. width. Electrophoresis was carried out at room temperature; the paper strips were dyed with bromphenol blue according to Kunkel and Tiselius (1951).

Buffer Solutions.—All buffers had *p*H 8.6 (glass electrode). The three buffers used in this investigation were (1) 0.05 M barbitone as described by Durrum (1950); (2) 0.2 M borate buffer, prepared by mixing 0.2 M boric acid (3.5 vol.) with 0.2 M sodium hydroxide (1 vol.); (3) borate-barbitone buffer prepared by mixing equal volumes of (1) with 0.1 M borate buffer. The latter was half strength buffer (2).

Results

In this investigation all runs were carried out with samples of the same serum from a normal adult female. Except for the ageing experiment (see below), the serum was stored at $-20°$ C. when not required. When electrophoresis was carried out soon after collecting the blood and preparing the serum, two distinct bands were observed in the β-globulin region when borate was the buffer used. This is illustrated in Fig. 1A. The strongest band (β_1) had a mobility about 0.4 that of the albumin as measured from the γ globulin band. The slower β-globulin band (β_2) was relatively fainter in colour and of a greyer shade than the β_1. In barbitone buffer, when electrophoresis was carried out immediately following the borate electrophoresis, separation of these two was much less clearly defined (Fig. 1B), the mobility of the β band being similar in this buffer to that of β_1 in borate. When samples of the serum were allowed to stand at room temperature for several hours, the β_2 band became much fainter in borate buffer and after 24 hours had disappeared. The electrophoretic pattern then only showed one β band and the disappearance of the slower component was accompanied by an increase in depth of colour of the remaining β_1 band. Refrigeration of the serum failed to stabilize the β_2 component, though it disappeared much more slowly than when the serum was kept at room temperature. After 10 days at $-20°$ C. the β_2 band was very faint. If the serum was aged sufficiently a third component (β_A), just faster and relatively fainter than the β_1 band, appeared. This is illustrated in Fig. 1C, which is the electrophoretic pattern in borate buffer of the serum after keeping for 48 hours at room temperature. In barbitone buffer, however, this aged serum gave only one β globulin band (Fig. 1D).

Short periods of electrophoresis were found necessary to demonstrate the presence of the slower, labile β_2 component in fresh serum. With prolonged periods, e.g., for 17 hours at 3 volts/cm., after which the albumin had travelled through a similar distance as in the shorter period at the higher voltage, it was no longer visible. However, the β_A band of the serum aged 48 hours was present under these conditions.

Other non-borate buffers of *p*H 8.6 were investigated; e.g., glycine-sodium hydroxide, citrate and phosphate; but these were similar to barbitone in their effect on the β-globulins. Borate buffer has

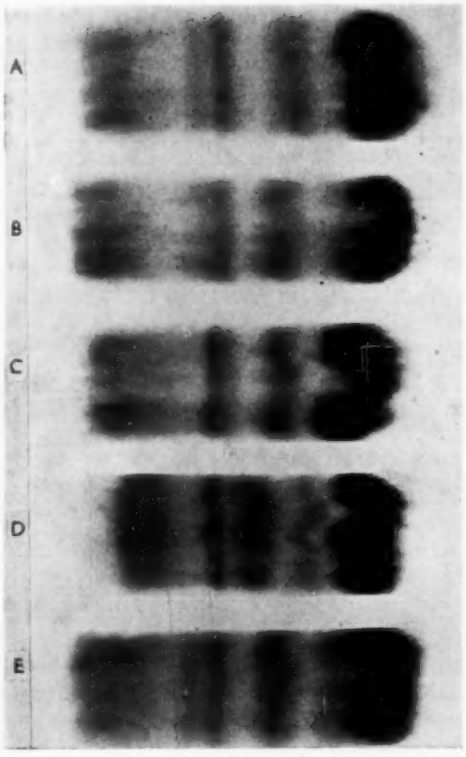

FIG. 1.—Paper electrophoresis of normal human serum. (A) Fresh serum in borate buffer, (B) the same in barbitone buffer, (C) 48-hour-old serum in borate buffer, (D) the same in barbitone buffer, (E) fresh serum in borate-barbitone buffer.

the disadvantage of increasing the mobility of α_1-globulin to that of albumin, but this was overcome by employing the mixed borate-barbitone buffer, which also differentiated the β components of fresh or aged serum as satisfactorily as borate alone. This is illustrated for the fresh serum in Fig. 1E. This electrophoresis was carried out on the day following the electrophoresis in barbitone, the serum in the meantime being stored at $-20°$ C.

Discussion

The mixed borate-barbitone buffer has been used for some considerable time in these laboratories for the routine examination of sera, and the double band as illustrated in Fig. 1E has always been present provided the sera are examined within one to two hours after the collection of the blood, or if stored at $-20°$ C. for a few days. Sera kept for more than several hours at room temperature generally give only one β band, but occasionally we have observed all three bands (β_1, β_2, and β_A). It is clear from these observations and from the experiments described above that the β_2 component is very labile and is rapidly converted, probably by enzyme action, to a protein of higher mobility. On the other hand the faster component (β_A) of the aged serum is stable and would seem to be an artefact derived from one of the β_1- or β_2-globulins. These changes would of course be taking place during the course of electrophoresis, which would explain the disappearance of the β_2 band after long periods of electrophoresis. Of the many photographs of paper electrophoresis strips of serum which we have seen, the great majority show only one β band. This is in agreement with our finding that the labile component is not well separated in barbitone buffer. However, we have observed a few examples which show distinct separation of the β_1, β_2 components in this buffer. For example these two bands were observed in pathological sera by Grassmann (1951) and Kawerau (1954) and in normal sera by Antonini and Piva (1953). Occasionally also the third component, similar to the artefact β_A, has been observed (cf. Kawerau, 1954), but the infrequency of these examples suggests that it is exceptional to obtain more than one definite β band in non-borate buffer. As far as our experiments are concerned, it would appear that borate ions are specifically concerned in altering the mobilities of some of the β-globulins. It is well known that borate ions can form complexes with substances containing α-glycol groups and therefore alter their electrophoretic mobilities. Groups of this type occur in many carbohydrates and lipids, and, since these (especially the latter) occur with the β-globulins, alteration of some of the components by borate might be expected. It is not clear, however, why the β_2 component should become slower, since borate complexing usually confers extra negative charges and therefore higher mobility, as is the case with the β_A component and α_1-globulin.

Owing to the transient nature of the β_2-globulin, it has not yet been possible to correlate any variations in its strength with any particular pathological condition, but this could well be of significance in certain cases, and any method, such as the use of borate, which improves separation in the highly complex β-globulin region is clearly desirable.

Summary

In borate buffer, one of the β-globulins of fresh, normal human serum has a lower electrophoretic mobility and after paper electrophoresis is separated

as a relatively faint band from the band of the other β-globulins. Separation in non-borate buffers is less satisfactory.

The component of lower mobility is converted, after a few hours at room temperature, to one with a mobility similar to that of the other β-globulins.

After sufficient ageing, one of the β-globulins is converted to a component which, in borate, travels somewhat ahead of the β-globulin band, but it is not separated in barbitone.

Borate-barbitone buffer combines the advantages of borate and barbitone.

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AMINO-ACID COMPOSITION OF SERUM PROTEINS IN HEALTH AND DISEASE

BY

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Electrophoresis of serum proteins in disease frequently discloses abnormalities. The alterations are rarely diagnostic; thus a diffuse increase of the γ-globulin is common to many clinically unrelated conditions. It would be of considerable value therefore if it were possible to distinguish between the proteins which are responsible for a common abnormality in the electrophoretic pattern. It was with a view to relating the composition of the serum proteins to disease that we have undertaken a study of the amino-acid composition of the electrophoretic fractions in liver disease, kidney disease, and myelomatosis, conditions in which it is well known that the serum proteins show great abnormality.

Existing data on the amino-acid composition of serum proteins are very meagre, and we have been unable to find any literature dealing with the amino-acid content of electrophoretically pure serum protein fractions. The composition of the plasma proteins in healthy subjects has been investigated by Brand, Kassell, and Saidel (1944), Shemin (1945), Brand (1946), and Brand and Edsall (1947), who give data for the percentage composition of certain amino-acids in albumin and various globulin fractions separated by precipitation methods. Brand and Edsall (1947) include results of complete amino-acid analyses of α- and β-globulins as well as of albumin and γ-globulin, but the fractions were not electrophoretically homogeneous. The findings have been reviewed by Marrack and Hoch (1949).

The content of a few amino-acids in plasma proteins has been studied in pathological conditions. Tuchman and Sobotka (1932) found discrepancies in Wu and Kjeldahl methods of serum protein determination in cases with nephrosis or nephritis with oedema, and concluded that in these conditions the albumin tyrosine content is increased while the globulin tyrosine is diminished. Alving and Mirsky (1936) studied the cystine content of the plasma proteins in eight cases of kidney disease and in five healthy individuals; part of the albumin fraction from patients with the nephrotic syndrome was found to have an abnormally low cystine content. Bálint and Bálint (1942 and 1943) estimated tyrosine, tryptophan, cystine, arginine, and histidine in the plasma proteins from patients with acute infections and in cases of anaemia. In acute infections they found tyrosine and tryptophan decreased, and cystine increased, in the albumin, and the reverse changes in the globulin. Emmrich (1950) investigated the cystine content of normal and pathological serum proteins and found abnormal levels in protein deficiency, cirrhosis of the liver, chronic kidney disease, tuberculosis, and other chronic inflammatory processes. Schuck (1950) found variation in the cystine and methionine content of the plasma proteins in pregnancy with abnormal levels in the toxaemia of pregnancy. Müting (1951), who estimated only methionine, showed that the level of this amino-acid in serum protein was lower than normal in parenchymatous liver disease and high in cases of the nephrotic syndrome. Schreier (1951) estimated biologically threonine, valine, leucine, isoleucine, phenylalanine, lysine, histidine, cystine, and methionine in serum albumin from four cases of the nephrotic syndrome and single cases of liver disease, Hodgkin's disease, leukaemia, and carcinomatosis, but found no significant variation from normal.

Clinical Material

Data are presented here of the composition of the serum proteins of six healthy young adults, six patients with the nephrotic syndrome, six with liver disease, and six with multiple myeloma. Of the patients suffering from liver disease, four had cirrhosis, one had subacute viral hepatitis, and one was a case of acute hepatic necrosis. Except for one patient with the nephrotic syndrome and one with cirrhosis, all the patients were adults, and all except two were attending University College Hospital. In each case the clinical features of the condition were typical and the diagnosis was supported by appropriate pathological investigations or confirmed at necropsy. The sera from all the

patients showed abnormalities in the electrophoretic pattern of the proteins; notably there was an increase of the γ-globulin and a decrease of the albumin in the cases of liver disease, an increase of the α_2-globulin and a decrease of the albumin and γ-globulin in those with the nephrotic syndrome, and a marked compact increase of the γ-globulin in the cases with myeloma.

Method

The proteins were fractionated by electrophoresis on filter paper, the fractions hydrolysed, their constituent amino-acids separated and identified by paper chromatography and then estimated colorimetrically. The α_1-globulin was not analysed because insufficient was available.

Electrophoresis.—The proteins were fractionated by paper electrophoresis as described by Flynn and de Mayo (1951). Serum, 0.2 ml., was applied to each of eight or 10 filter paper strips (Whatman No. 31 extra thick) and a potential of 120 volts applied for 18 hours. The strips were heated at 80° C. until just dry before staining with bromphenol-blue.

Isolation of Proteins.—The protein fractions so detected were cut out and steeped in approximately 150 ml. of 0.5 N-sodium hydroxide for 24 hours with occasional shaking. The solutions were filtered, and the filtrate adjusted to *p*H 5 with sulphuric acid. Mercuric chloride, 1 g., was added and the whole heated until just boiling. The coagulated proteins were allowed to sediment for 24 hours and the supernatant fluid decanted as completely as possible. The remaining sludge was transferred to a centrifuge tube, being washed in with water, and the proteins collected by centrifuging; they were then washed twice by mixing with water and re-centrifuging.

Preparation of the Hydrolysate.—To the protein in each centrifuge tube 5 ml. of 6 N-hydrochloric acid was added. The tube was sealed and incubated at 100° C. for 15 hours. The hydrolysate was then evaporated to dryness under reduced pressure and the residue dissolved in 2 ml. of 10% (v/v) iso-propyl alcohol as recommended by Block (1950). This solution was desalted electrolytically and subsequently a sample taken for a total nitrogen determination by a micro-Kjeldahl procedure.

Chromatography.—Two-way chromatography was carried out as described by Dent (1948) using Whatman No. 4 filter paper. A volume of hydrolysate equivalent to 0.1 mg. of nitrogen was applied to each chromatogram. The developed chromatograms were lightly sprayed with 0.2% (v/v) ninhydrin in n-butanol and allowed to stand at room temperature until the colour developed; usually they were left overnight.

Determination of Amino-acids.—From each chromatogram the amino-acid spots were cut out with a margin of unstained paper, together with paper blanks of the same size. In practice all the amino-acid spots could often be contained within circles of 1½ in. diameter and then a single blank of the same dimensions would be used. The cut-out amino-acid spots were transferred to test-tubes and 1 ml. of 5% (v/v) ninhydrin in 90% iso-propyl alcohol (Block, 1950) added to each. The tubes were shaken well and incubated in a water bath at 80° C. for 10 minutes. After cooling, 5 ml. of 70% (v/v) acetone in water was added to each tube. The tubes were shaken to dissolve the dye and then stood for 20 minutes to complete the solution (Fowden, 1951). Certain of the amino-acids gave too intense a colour for convenient reading in the photometer; in these instances further dilution with 70% acetone was carried out. The optical densities were measured at 570 mμ. The chromatography and amino-acid estimations were carried out in duplicate. Isoleucine and leucine were estimated together, since their separation cannot be effected with the solvents used. Proline was not estimated as the recovery was very poor, and in the case of cystine and other amino-acids the amount was too small to determine.

Calculation.—Factors were obtained correlating the colorimetric readings with the amounts of individual amino-acids present by subjecting proteins of known composition to the above procedure. Casein, gelatine and electrophoretically pure normal serum albumin were used for this purpose. The data given by Hawk, Oser, and Summerson (1947) for casein and gelatine, and by Brand and Edsall (1947) for serum albumin, were used for the calculation of the factors; the data given by these authors were first corrected to express the percentage amino-acid composition per 100 g. amino-acids. By this method of calculation allowance is made for variations in the amount of colour produced in the reaction with ninhydrin, as well as for any lack of quantitative recovery of the individual amino-acids. The colorimetric readings were multiplied by the appropriate factors and the values thus obtained for the actual amounts of individual amino-acids present were then expressed as a percentage of the total amount of amino-acids estimated.

Reproducibility.—The final figure for each individual amino-acid value was shown to be reproducible to within 10% for all the acids except lysine and histidine, which were reproducible to within 15%, and for arginine which was reproducible to within 20%. The amount of arginine detected appears to be related to the length of time required for desalting; if this is prolonged arginine is converted to ornithine (Stein and Moore, 1951).

Results

The results are shown in Figs. 1–12, and values for the average composition of the normal serum proteins are given in Table I.

Table I shows that the different fractions differ somewhat in composition, the content of serine, alanine, threonine, and lysine being particularly variable. These findings are in accord with those of other authors using different methods of analysis (Brand and Edsall, 1947).

Analysis of the results from the pathological sera shows that in no case are the serum proteins grossly

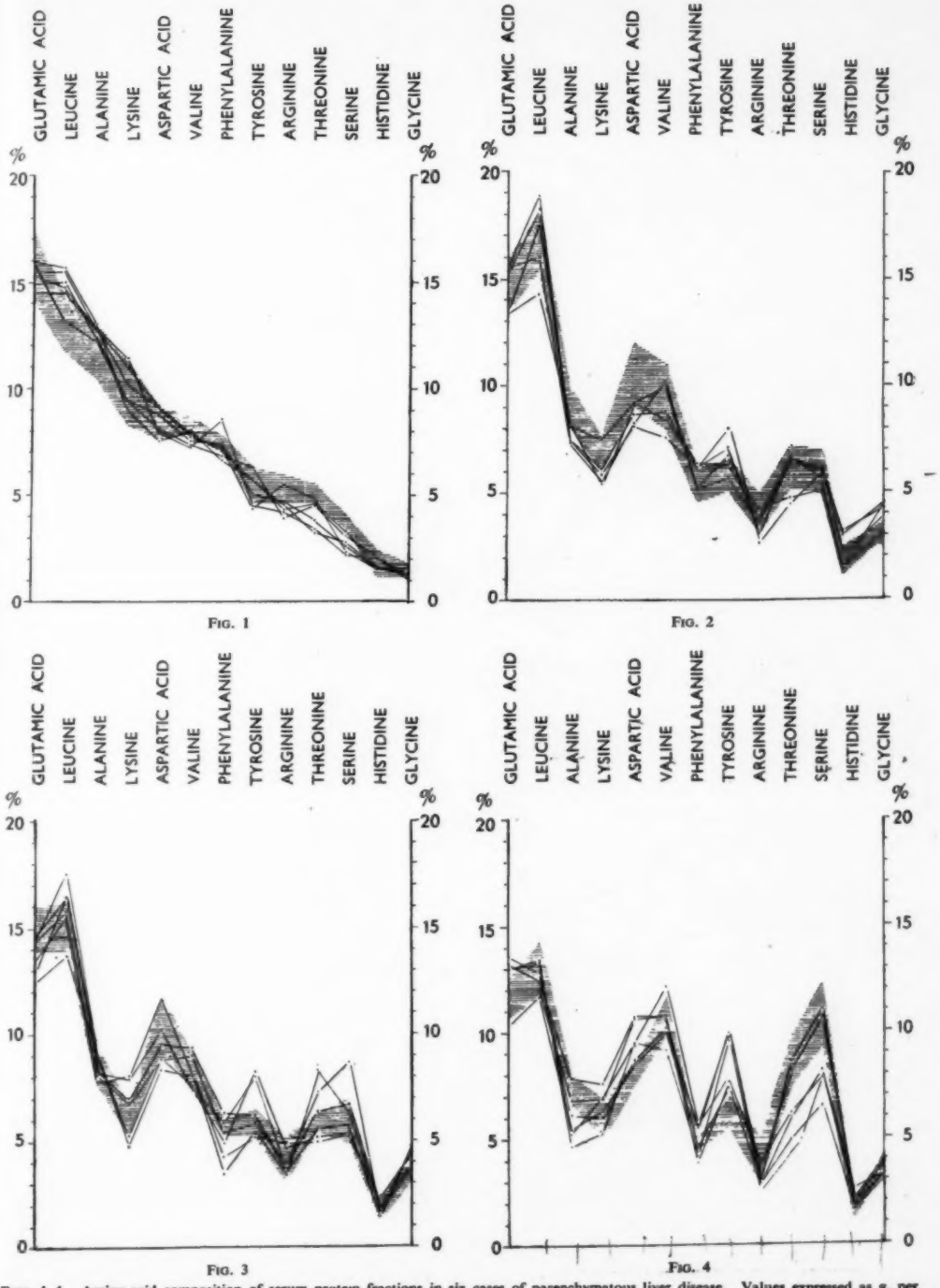

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIGS. 1–4.—Amino-acid composition of serum protein fractions in six cases of parenchymatous liver disease. Values expressed as g. per 100 g. amino-acid estimated. Shaded area represents range of values found in six normals. Fig. 1 is for albumin, Fig. 2 for α_2-globulin (five cases only), Fig. 3 for β-globulin, and Fig. 4 for γ-globulin.

FIG. 5

FIG. 6

FIG. 7

FIG. 8

FIGS. 5–8.—Amino-acid composition of serum protein fractions in six cases of nephrotic syndrome. Values expressed as g. per 100 g. amino-acid estimated. Shaded area represents range of values found in six normals. Fig. 5 is for albumin, Fig. 6 for α_2-globulin, Fig. 7 for β-globulin, and Fig. 8 for γ-globulin.

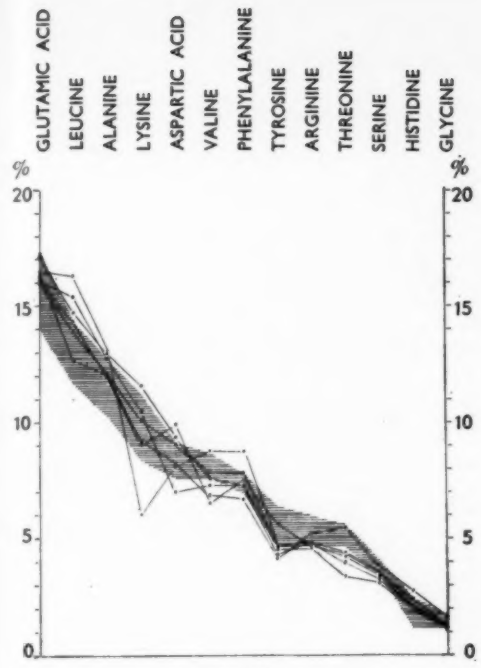

FIG. 9

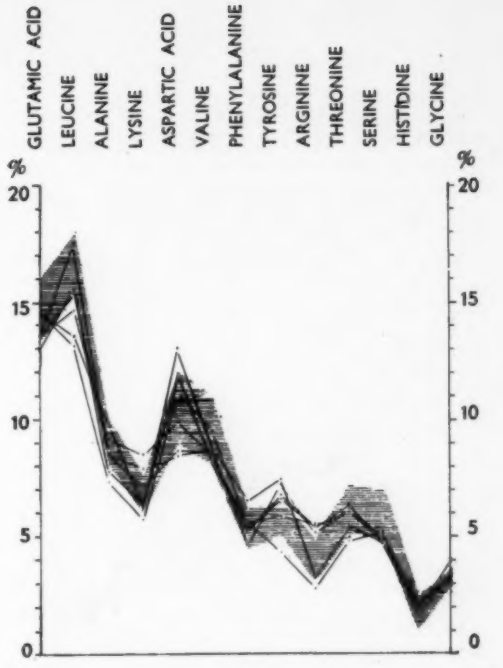

FIG. 10

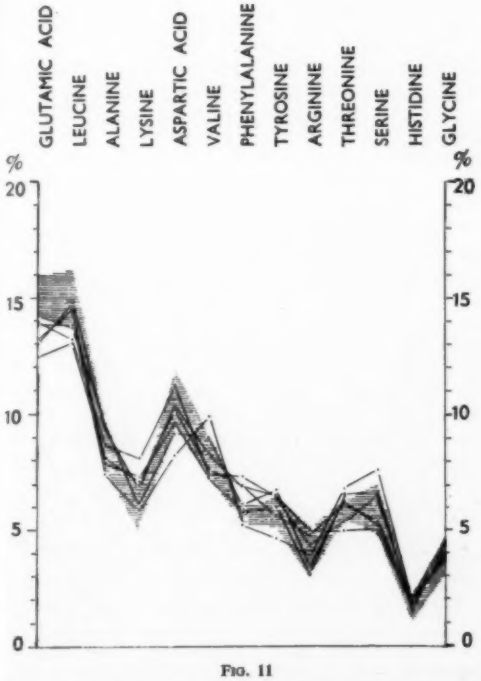

FIG. 11

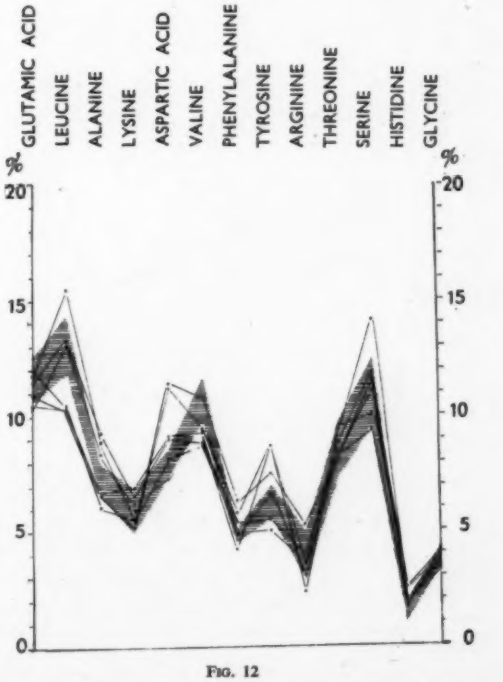

FIG. 12

FIGS. 9–12.—Amino-acid composition of serum protein fractions in six cases of multiple myeloma. Values expressed as g. per 100 g. amino-acid estimated. Shaded area represents range of values found in six normals. Fig. 9 is for albumin, Fig. 10 for α_2-globulin, Fig. 11 for β-globulin, and Fig. 12 for γ-globulin.

TABLE I

MEAN VALUES* FOR THE COMPOSITION OF NORMAL SERUM PROTEINS

	Albumin	α_2-globulin	β-globulin	γ-globulin
Glutamic acid	15·9	14·1	15·1	11·8
Leucine ..	13·0	16·5	14·7	12·8
Alanine ..	11·6	9·4	8·7	6·6
Lysine ..	9·4	6·0	6·1	6·0
Aspartic acid ..	8·4	11·0	10·6	8·1
Valine ..	8·3	9·2	8·8	10·5
Phenylalanine	7·5	5·5	5·1	4·7
Tyrosine ..	5·1	5·6	5·8	5·6
Arginine ..	5·3	3·4	4·3	3·9
Threonine ..	5·0	6·3	5·8	8·4
Serine ..	3·7	5·3	6·0	10·8
Histidine ..	2·2	1·7	1·5	1·5
Glycine ..	1·5	3·4	3·7	3·9

* Values expressed as g. per 100 g. amino-acid estimated.

abnormal in composition, at least in terms of those amino-acids estimated. More critical examination of the data, however, shows that minor variations may occur in individual cases. In Table II the frequency and direction of deviations from the normal range which are considered to be definitely outside the range of experimental error are indicated.

Discussion

Paper electrophoresis of proteins has several advantages over the classical Tiselius technique, an important one being that all the fractions are entirely separated from one another in a single operation. This technique therefore provided us with a simple method of isolating electrophoretically pure protein fractions for the purposes of this study.

Abnormality of the chemical composition of the serum proteins might be expected in disease, since the proteins often show considerable variation from normal in their physical properties. Some of these changes, however, may result from an alteration in the structure of the protein molecules rather than from an abnormal chemical composition. A recent report shows that great deviation from normal may occur in the chemical composition of proteins under artificial circumstances: thus, in infants fed on lysine-deficient diets, proteins appeared in which lysine was replaced by arginine (*Lancet*, 1953).

Our findings establish that there is no gross deviation in the proportions of at least 13 amino-acids in the serum proteins, even when there is a marked disturbance of protein metabolism. The minor changes we have found must be interpreted with caution. The fact that data are available from only six normals must be kept in mind, and it is this in particular that has prevented statistical analysis of the results. It must also be noted that when the percentage of one amino-acid is increased the increase occurs at the expense of others which must therefore be decreased. The experimental error is regrettably large and indeed estimation of some of the amino-acids present in smaller quantity was abandoned because of this; however, as noted in Table II, some of the results fall well outside this error. Despite all these reservations our findings in the pathological sera require some comment.

The results show more frequent deviations from normal composition in the cases of liver disease than in the other conditions studied. It is also of interest that in liver disease and myelomatosis abnormalities occurred most often in the γ-globulin fraction, which was the fraction showing the greatest increase in concentration. In the cases of the nephrotic syndrome, however, although the α_2-globulin showed the greatest increase, abnormalities of composition were found more frequently in the β-globulin.

Variations in the composition of the serum protein fractions in disease may result either from the production of proteins of abnormal composition or

TABLE II

FREQUENCY OF DEVIATION OF AMINO-ACID COMPOSITION FROM THE NORMAL RANGE

	Liver Disease				Kidney Disease				Multiple Myeloma			
	Albumin	α_2	β	γ	Albumin	α_2	β	γ	Albumin	α_2	β	γ
Leucine					↑							
Alanine				↓			↓↓					
Aspartic acid				↑↑		↓	↓					↑↑
Phenylalanine			↓			↑	↑↑	↑				↑
Tyrosine		↑	↑↑	↑↑↑				↓				↑↑
Threonine	↓↓		↑	↓↓					↓			
Serine	↓↓↓		↑	↓								
Glycine		↑↑↑						↑				

↑ = Increase and ↓ = decrease in concentration. Number of arrows corresponds to number of cases showing abnormality.

from alteration in the relative proportions of proteins normally present. With regard to the latter possibility it is probable that many different proteins together make up the electrophoretic spectrum of serum, and since the major bands of the spectrum show differences in chemical composition it is likely that the different proteins which together constitute a particular fraction will also differ slightly in composition. If this is so, then a marked increase or decrease of the concentration of a particular component protein could cause the overall composition of that fraction to differ from normal. Variation in the proportion of component proteins could be held responsible for the small variation in composition found in the serum proteins of healthy individuals.

In all the cases of liver disease studied, electrophoresis showed a diffuse increase of the serum γ-globulins, the increase affecting the faster moving γ_1-globulins to the greatest extent. The chemical abnormalities found in the whole fraction might therefore be due to a selective increase in the γ_1-globulins. On the other hand this finding, and the abnormalities found in the other fractions, could be the result of synthesis of abnormal proteins.

In the nephrotic syndrome the predominant proteins to appear in the urine are albumin and α_1- and β-globulins (Slater and Kunkel, 1953; Squire, 1953; Flynn, 1954). This differential loss of protein can be related to the molecular weight of the proteins (Squire, 1953), and may serve to explain why we encountered variation in protein composition most frequently in the β-globulin. The normal serum β-globulin fraction is composed of two main groups of proteins, one with a molecular weight of around 90,000, the other with a molecular weight of about 1,300,000. Differential loss of the lower molecular weight component, should it differ somewhat in amino-acid composition from that of the other component, could then be responsible for the serum β-globulin appearing abnormal in composition. Lack of abnormality in the serum albumin could be attributed to all the albumin components passing into the urine with equal facility. The findings of Albanese, Davis, Smetak, and Lein (1949) suggested that the albumin lost in the urine in the nephrotic syndrome may differ in composition from normal plasma albumin. Their results, taken in conjunction with our finding that the serum albumin is normal in composition, suggest that abnormal albumins are synthesized but are immediately excreted in the urine. However, it must be noted that Schreier's (1951) results on the urine from two cases do not confirm the findings of Albanese *et al.* (1949).

In myelomatosis the presence of a chemically abnormal serum protein might well have been expected in view of the findings of Dent and Rose (1949) that Bence-Jones protein entirely lacks methionine. However, our findings with 13 amino-acids show little alteration in the composition of the γ-globulin fraction despite its great increase in concentration.

Taking all the above results into consideration one may conclude that amino-acid analysis of serum protein fractions is unlikely to be of diagnostic value.

Summary

The amino-acid composition of electrophoretically homogeneous serum protein fractions has been studied in six healthy young adults and in six cases each of liver disease, the nephrotic syndrome, and myelomatosis. Thirteen amino-acids were estimated quantitatively.

No gross or characteristic alteration from normal was found in the pathological sera in the albumin, α_1-, β-, and γ-globulins.

Minor deviations from normal composition were found in individual cases. The possible significance of these findings is discussed.

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PLASMA CREATININE

BY

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The non-specificity of reactions for creatinine is a well-recognized drawback in most methods for its determination. The Jaffé colour reaction which forms the basis of most procedures is given by numerous substances other than creatinine, over 30 being listed by Hunter (1928). They include, apart from substances related to creatinine, acetone, aceto-acetic acid, and pyruvic acid.

Various methods of estimating true creatinine as opposed to the total Jaffé-positive chromagen have been proposed. Miller and Dubos (1937), using suspensions of a creatininase-producing bacterium, carried out the Jaffé reaction on plasma filtrates before and after incubation with a suspension of the organism, and thus calculated the true creatinine by difference. According to this technique, the true plasma creatinine constituted 80–100% of the total chromagen. Methods employing adsorption on Lloyd's reagent have also been claimed to give true creatinine values. This substance is a hydrated aluminium silicate. In acid solution it adsorbs creatinine which can subsequently be eluted by alkali.

Recently Koštíř and Šonka (1952) published a method for creatinine determination based on the observation that pyruvic acid is a Jaffé-positive substance which can be oxidized by ceric sulphate without destroying creatinine. Normal values found by this method were from 0.3 to 0.6 mg./100 ml. with a mean of 0.48 mg./100 ml. A series of determinations with ceric sulphate showed a reduction of 60% on a parallel series without ceric sulphate treatment. In our hands normal plasmas (11) treated with ceric sulphate by this method gave a range of 0.77 to 1.21 mg./100 ml., with a mean of 0.97 mg./100 ml. This was a reduction of 16% on the values obtained on the same plasmas without ceric sulphate treatment. This reduction was significant but was not of the magnitude found by Koštíř and Šonka.

We have also investigated the adsorption procedure with Lloyd's reagent (Fuller's earth) of Haugen and Blegen (1953) as modified by Owen, Iggo, Scandrett, and Stewart (1954), who found good agreement between the Lloyd's adsorption method and the specific enzymatic method. It was considered that another approach to checking its specificity would be to investigate the Lloyd's adsorbed material chromatographically. This method has given results which are lower than those obtained with the ceric sulphate method; and chromatograms of the eluates from the Lloyd's adsorption have shown only one Jaffé-positive spot, identical with that for creatinine. We conclude that the Lloyd's procedure is, in fact, specific for creatinine in plasma.

Method

Reagents.—The following are required:—

Sodium Tungstate.—10%.

Sulphuric Acid.—⅔ N.

Lloyd's Reagent.—Fuller's earth B.D.H.: Haugen and Blegen (1953) point out that not all samples of Fuller's earth are suitable. Each batch should be tested for satisfactory adsorption.

Oxalic Acid.—Saturated solution.

Sodium Hydroxide.—2.5 N-NaOH.

Picric Acid.—Saturated solution.

Alkaline Picric Solution.—Picric acid solution (27.5 ml.) plus 2.5 N-NaOH (5.5 ml.) made up to 100 ml. with water; prepared freshly for use.

Standard Creatinine Solution.—1 mg./100 ml. in water.

Procedure.—To 3 ml. of plasma is added 4.5 ml. of water, 1.5 ml. of sodium tungstate, and 3 ml. of ⅔ N sulphuric acid. After thorough mixing and standing for 30 min., the mixture is filtered through a No. 1 Whatman filter paper.

To 100 mg. (±2 mg.) Lloyd's reagent in a centrifuge tube is added 5 ml. of filtrate (equivalent to 1.25 ml. plasma) and 0.5 ml. of saturated oxalic acid. The tube is stoppered and shaken intermittently for 10 min. (As an alternative to weighing individual lots of the Lloyd's reagent, a suspension of 10 g. in 100 ml. of water may be made and 1 ml. of this solution taken after thorough shaking.) The tube is then centrifuged at 3,000 r.p.m. for 20 min. The supernatant is decanted and the tube allowed to drain.

To the packed deposit is added 7.5 ml. of freshly prepared alkaline picrate solution. The deposit is stirred, the tube stoppered and shaken intermittently for

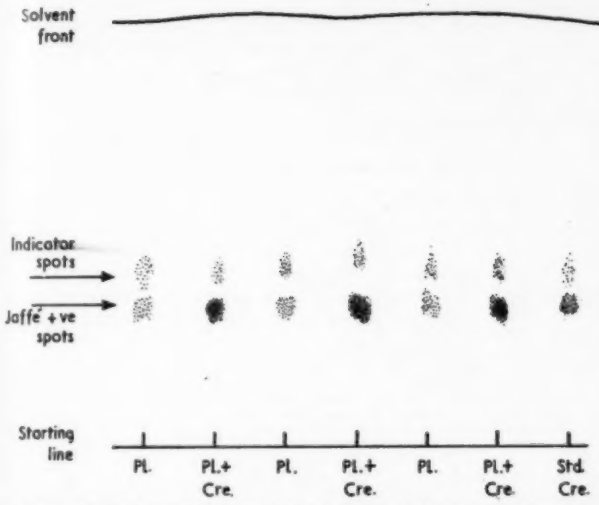

FIG. 1.—Chromatogram of eluates of Lloyd's adsorbed plasma (Pl.), plasma plus creatinine (Pl.+Cre.), and of creatinine standard solution (Std. Cre.). Run for 16 hours in butanol-acetic acid-water solvent, then sprayed with alkaline picrate solution.

10 min. It is centrifuged for 10 min., and then placed in a water bath (20°–24° C.) and allowed to reach the bath temperature.

The solution is then read in a spectrophotometer at a wavelength of 520 mμ. Satisfactory results have also been obtained with the Ilford bright spectrum green light filter No. 624 in a photo-electric colorimeter.

A blank consisting of 5 ml. of water, and two 5 ml. standards containing 0.01 mg. and 0.025 mg. (equivalent to 0.8 mg. (0.01 × 100/1.25) and 2 mg. creatinine/100 ml. plasma), are prepared in a similar manner, i.e., treated with Lloyd's reagent and alkaline picrate like the filtrate.

Experiments

Elution of Creatinine and Chromatography.—Plasma was deproteinized, the filtrate allowed to stand in contact with Lloyd's reagent, the latter then centrifuged down and the tube drained. Sodium hydroxide, however, could not be used for elution as this interferes with obtaining satisfactory chromatograms. Hence other alkalis which could be removed before running the chromatogram were investigated. Ammonium hydroxide and potassium hydroxide proved unsuitable. A saturated solution of barium hydroxide, i.e., approximately N/3, was satisfactory. This was subsequently exactly neutralized by sulphuric acid using methyl orange as indicator. The precipitate of barium sulphate was spun down, the supernatant decanted and evaporated to dryness at reduced pressure at a temperature not exceeding 37° C. It was then taken up in 0.1 ml. of distilled water and transferred as completely as possible to chromatogram paper (No. 1 Whatman).

With each sample of plasma a second sample of the same plasma, with added creatinine, was run. The creatinine (10 μg. in 1 ml. of water) was added to the 5 ml. of filtrate, and adsorbed on Lloyd's reagent.

The chromatograms were run 16 hours in butanol-acetic acid-water solvent (in the proportions 4:1:5) and then sprayed with a freshly mixed alkaline picrate solution (equal parts of a 3% NaOH solution and a saturated solution of purified picric acid dilution in the proportion of 7.5 to 2.5 ml. of water).

Results

Ten normal plasmas, each as plasma alone and as plasma plus creatinine, showed a single Jaffé-positive spot. That for plasma plus creatinine was considerably darker and of the same Rf value as the Jaffé-positive spot for plasma alone. The methyl orange indicator (contained in the test solutions) ran in advance of the Jaffé-positive spot.

It is considered that the results of these chromatograms indicate that Lloyd's reagent will adsorb

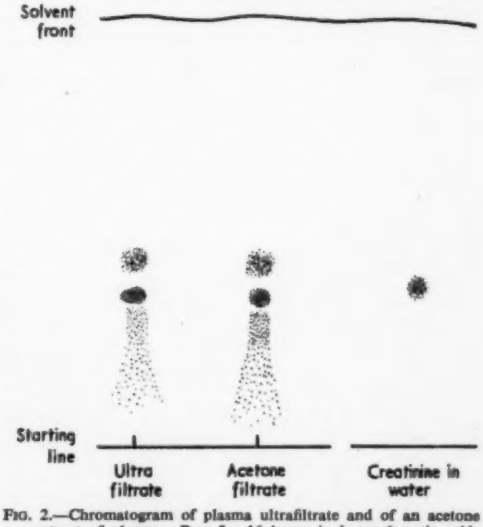

FIG. 2.—Chromatogram of plasma ultrafiltrate and of an acetone extract of plasma. Run for 16 hours in butanol-acetic acid-water solvent, then sprayed with alkaline picrate solution. Two Jaffé-positive spots were discerned in plasma together with streaking—"salt effect."

creatinine alone of the Jaffé-positive substances present in plasma (Fig. 1).

Chromatograms were also run on ultrafiltrates of plasma and on plasma deproteinized with acetone. In these, salts are of course present and these interfere with the obtaining of clear-cut chromatograms. However, at least two spots were detectable (Fig. 2). Attempts at de-salting without destruction of the creatinine were unsuccessful.

TABLE I

PLASMA CREATININE VALUES BY DIFFERENT PROCEDURES (MG./100 ML.)

A "Total" (Untreated Plasma)	B After Ceric Sulphate	Reduction (% B on A)	C Treated with Lloyd's Reagent	Reduction (% C on A)	D Treated with Lloyd's and Ceric Sulphate	Reduction (% D on A)
1·29	1·13	12	0·86	33	0·82	36
1·27	1·00	21	0·77	39	0·78	39
1·34	1·00	25	0·91	32	0·92	31
1·11	0·92	17	0·80	28	0·84	24
1·08	0·94	13	0·80	26	0·76	30
1·21	1·00	17	0·92	24	0·90	26
1·18	0·98	17	0·80	32	0·84	29
1·35	1·10	19	0·95	30	1·07	21
1·04	0·90	13	0·70	33	0·74	29
1·10	0·91	17	0·86	22	0·80	27
Average 1·20	0·99	17	0·84	30	0·85	29

As another method of checking the specificity of the Lloyd's adsorption technique, the material adsorbed on Lloyd's reagent after elution by alkali and subsequent neutralization of the alkali was then treated with ceric sulphate. For this purpose, tests of 10 normal plasmas were prepared in parallel in four different ways: (A) Untreated with either ceric sulphate or Lloyd's reagent, i.e. total plasma creatinine; (B) treated with ceric sulphate; (C) treated with Lloyd's reagent; (D) treated with Lloyd's reagent followed by ceric sulphate.

No further reduction in the plasma creatinine value was obtained (Table I).

Summary and Conclusions

An investigation of certain methods of plasma creatinine determination is described. Treatment with ceric sulphate was found to give a slight reduction only of the "total" creatinine. The Lloyd's reagent adsorption procedure was investigated chromatographically and only one Jaffé-positive substance, identical with that of creatinine similarly treated, was obtained.

It is concluded that the Jaffé-positive material measured by the Lloyd's adsorption method is creatinine and only creatinine. It appears that ceric sulphate treatment does not completely eliminate non-specific Jaffé-positive material.

I would like to thank Professor E. J. King and Dr. C. E. Dalgliesh for their help during these investigations, and Mr. D. Simmonds for the illustrations.

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J. clin. Path. (1955), **8**, 163.

AN ASSESSMENT OF NEW TESTS FOR DETECTING BILIRUBIN IN URINE

BY

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Recently a simple tablet test for detecting bilirubin in urine has been introduced, and since this test is claimed to have considerable advantages over existing ones used by clinicians we have compared it with others commonly used to detect bilirubinuria. A comparison has been made of the tablet test ("ictotest") with the Harrison-Fouchet and iodine ring tests—selected because they are so commonly used—as well as with a modified Fouchet test which will be referred to as the mat Fouchet test. The last is very simple and quick, and unlike the standard Harrison-Fouchet test is suitable for routine use in the consulting room. The sensitivity of the tests has been compared and the effect of urobilinogen, drugs, and other interfering substances noted.

In the "ictotest" bilirubin is concentrated by adsorption on a cellulose asbestos mat and then allowed to couple with a diazo compound, in tablet form, to give a coloured complex. It has been tried in America by Klatskin and Bungards (1953), Sobotka, Luisada-Opper, and Reiner (1953), Giordano and Winstead (1953), and by Free and Free (1953), and in this country by Tallack and Sherlock (1954). All these authors have commented favourably on its simplicity, sensitivity, and specificity. As we also consider that the "ictotest" and the mat Fouchet test are superior to the crude iodine test still used by many, we record our findings in the hope that we may encourage their general adoption.

Material

Eighty consecutive urine specimens, sent to the laboratory in connexion with other liver function studies, were tested, as well as 10 specially selected because they were normal, contained salicylates, or had a very high concentration of bilirubin.

Methods

The urines were submitted to some or all of the following tests:—The "ictotest," mat Fouchet, Harrison-Fouchet, iodine ring, Schlesinger test for urobilin, Obermayer test for indican, ferric chloride test for salicylate, and the heat test for protein. All these tests, with the exception of the first two, were carried out as described by Harrison (1947), and because of the known instability of bilirubin in urine (Klatskin and Bungards, 1953) all were performed as soon as possible on the day the specimens were received. Positive results were assessed on a rough quantitative basis, being graded as strong, moderate, or weak; doubtful positives were recorded as such. Ease of assessment and especial difficulties were also noted.

The "ictotest" and the mat Fouchet test were carried out as follows:

"Ictotest."—The tablets and mats manufactured by the Ames Company were used. Each tablet contains 0.2 mg. p-nitrobenzene diazonium p-toluene sulphonate, 100 mg. sulphosalicylic acid, 10–20 mg. sodium bicarbonate, and 15–25 mg. boric acid. The mats measure about 20 × 20 × 4 mm. and consist of a cellulose asbestos mixture. Five drops of urine were allowed to soak into a test mat and a tablet was then placed on the moistened area. One drop of water was placed on top of the tablet and allowed to remain for a few seconds before a second drop was added, carrying the fluid on to the test mat below. The result was read within 30 seconds; the development of a purple colour on the mat was recorded as positive and all other colours, and any change after 30 seconds, were recorded as negative. The speed of development and intensity of the purple were used to assess the concentration of bilirubin.

Mat Fouchet Test.—Five drops of urine were allowed to soak into an "ictotest" mat and one drop of Fouchet reagent was added in the centre. The result was recorded as positive if a bluish colour developed within a few seconds and as negative if purple or pink colours appeared or there was just blanching of the yellow background. Positive results were graded according to the depth of colour produced.

The manufacturers state that either surface of the "ictotest" mat may be used. We found that using the original upper and lower surfaces of mats after splitting them in the horizontal plane in no way reduced the sensitivity of the test. Having performed many tests in parallel the use of half-mats was adopted for the remainder of the trial.

Attempts were made to find an alternative to the "ictotest" mat. Asbestos filter pads, filter paper of various grades, cellulose wadding, and the Franklin

barium chloride-impregnated plaster of paris tablet (Franklin, 1949) were tried, but all except the last were found inferior to the "ictotest" mat. The Franklin tablet was found slightly less efficient in adsorbing bilirubin on to the surface, but the smooth, pure white background was found advantageous when reading the result.

Results

Sensitivity of Tests.—Parallel tests on the 80 urines from patients with suspected liver impairment gave the results shown in the table. This shows that the Harrison-Fouchet and mat Fouchet tests give similar results, that the "ictotest" is considerably less sensitive than these, and that the iodine test is very unsatisfactory. The number of doubtful positives, which reflects the difficulty of assessing the result of a test, shows that the "ictotest" and the mat Fouchet test are often difficult to read, but even the Harrison-Fouchet test is not free from this objection.

TABLE
RESULTS OF TESTS FOR BILIRUBINURIA IN 80 PATIENTS WITH SUSPECTED LIVER DYSFUNCTION

Result of Test	Test			
	Harrison-Fouchet	Mat Fouchet	"Ictotest"	Iodine Ring
Negative	15 urines	14 urines	28 urines	68 urines
Doubtful positive	12 ,,	16 ,,	19 ,,	1 ,,
Weak ,, ..	31 ,,	27 ,,	14 ,,	—
Moderate ,, ..	7 ,,	9 ,,	6 ,,	—
Strong ,, ..	15 ,,	14 ,,	13 ,,	11 ,,
Total number of definite positives	53 ,,	50 ,,	33 ,,	11 ,,

To make a better assessment of the relative sensitivity of the four tests, serial dilutions were made with distilled water of six urines from markedly jaundiced patients. The results of the tests on these specimens showed that in each case the Harrison-Fouchet method detected bilirubin in the greatest dilution; in two cases down to 1 in 128. The Harrison-Fouchet test detected bilirubin at twice the dilution giving a positive mat Fouchet test and three times that giving a positive "ictotest." In all cases the iodine test was found to be markedly less sensitive than the other tests.

Effect of Interfering Substances.—Previous experience had shown that the presence of urobilin, indican, and salicylates in urine occasionally caused difficulty in interpreting the Harrison-Fouchet test, so the effect of these substances on the three more sensitive tests was investigated. Our results (see below) showed that each may be associated with difficulty when only small amounts of bilirubin are present. The effect of proteinuria on the "ictotest" was also noted.

Urobilin.—Of the 80 urines tested, 18 had a slight excess, nine a moderate, and nine a marked excess of urobilin. Analysis of the results showed that the relative sensitivities of the bilirubin tests were the same regardless of the concentration of urobilin. Therefore, as far as difficulties caused by urobilin are concerned the tests appear of equal status. In some cases where excess urobilin was present a brownish purple spot developed in the Harrison-Fouchet test and a red colour in the "ictotest."

Indican.—Of 54 urines tested for indican, 14 had abnormally large quantities. In these 14 urines the bilirubin tests showed agreement in all except four. In each of the latter an intense red colour was produced in the "ictotest," and in three a purplish brown spot in the Harrison-Fouchet test.

Salicylate.—Of 23 urines tested for the presence of salicylate, five gave positive ferric chloride tests which were shown conclusively to be due to salicylate. The tests for bilirubin on these five urines gave more strongly positive results with the mat Fouchet test than with the Harrison-Fouchet test. That this was attributable to salicylate was shown by testing two specimens of urine from non-icteric patients receiving large doses of salicylate —calcium salicylate and sodium *p*-amino salicylate (P.A.S.). The results showed that, not only does salicylate cause strong false positives with the mat Fouchet test, but may sometimes cause difficulty in interpreting the "ictotest," for a doubtful positive was recorded in the case of the patient receiving P.A.S. In the mat Fouchet test salicylates may produce a colour indistinguishable from that due to bilirubin, but sometimes the spot has a pinkish hue or a red halo surrounds a bluish centre; either of the latter findings should arouse suspicion of a false positive. In the "ictotest" P.A.S. therapy may account for a permanganate colour developing within 30 seconds; earlier development of an immediate orange-yellow (P.A.S.) or red colour (calcium salicylate) should arouse suspicion of a false positive. In the Harrison-Fouchet test salicylates cause a greyish-red spot quite unlike the bluish-green of a positive test for bilirubin; the appearance of a pink colour when a drop of Fouchet reagent is allowed to fall on the filter paper where there is no precipitate will serve to confirm that salicylates are present.

Protein.—Klatskin and Bungards (1953) stated that protein lowered the sensitivity of the "ictotest," and caused the production of a pink colour. Tallack and Sherlock (1954) did not confirm this and analysis of our results does not show a higher

incidence of proteinuria in urines giving a red colour with this test.

Ease of Assessment of Tests.—In most cases there was nothing to choose between the tests as regards ease of reading. On 30 occasions when a definite preference was noted, the Harrison-Fouchet test was preferred in 25, the mat Fouchet test in three, and the "ictotest" in two.

Discussion

Our results as regards the comparative sensitivity of the tests agree with those of other workers in pointing to the relative insensitivity of the iodine test. Our findings differ from those of Tallack and Sherlock (1954), however, in placing the "ictotest" as less sensitive than the Harrison-Fouchet and even the mat Fouchet test. In this connexion it is unfortunate that we were unable to compare the tests on a larger number of urines.

Considering the specificity of the tests the "ictotest" would appear to be very satisfactory, for on only three occasions in 180 tests were apparent false positives recorded, and even then the results were graded as doubtful positives in two. This very low incidence of false positives with the "ictotest" is in accord with the findings of previous workers. Turning to the occurrence of false negatives, however, the masking of positive tests by other intense colours occurs particularly with the "ictotest," though even the Harrison-Fouchet test is not free of this difficulty. Such was seen in some urines which contained excess urobilin and indican, although the nature of the substance responsible remains uncertain. False positives due to salicylates can occur with the mat Fouchet test and this represents an important drawback to this method.

When sensitivity, specificity, and ease of assessment are all taken into account, there would seem to be no case, in the laboratory, for replacing the Harrison-Fouchet test by the newer tests. In the choice of test for routine use by the clinician, however, where its technical simplicity has led to the general adoption of the iodine test, the advantages of the new tests would seem to justify a change. There is little to choose between the "ictotest" and the mat Fouchet test for this purpose. Both require little equipment; both use reasonably stable reagents, although that for the mat Fouchet is liquid; both can be performed in a few seconds, and the higher sensitivity of the mat Fouchet test is offset by its greater liability to yield false positives.

Summary

A tablet test for bilirubinuria has been compared with the Harrison-Fouchet and iodine tests, and also with a simple modification of the Fouchet test.

The Harrison-Fouchet test is considered to be the best, but the new tests have been shown to be greatly superior to the iodine test.

For routine use outside the laboratory it is recommended that the iodine test be replaced by one or other of the new tests.

We wish to thank the Ames Company, Ltd., for providing the tablets and test mats used in the trial, and Professor M. Maizels for helpful criticism.

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ADDENDUM

Since this paper was submitted for publication we have had the opportunity of performing over 200 tests with three other batches of "ictotest" reagents, using both whole and half-mats. Results on 26 routine specimens, and also on serial dilutions of urine containing much bilirubin, have confirmed our previous findings as to the relative sensitivities of the tests.

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UPTAKE OF SULPHATE ^{35}S BY LEUKAEMIC HUMAN BONE MARROW *IN VIVO*

BY

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A previous communication has reported the specific uptake of ^{35}S sulphate by the myeloid cells of human bone marrows *in vitro* (Lajtha, Ellis, and Oliver, 1953). Experiments *in vivo* on rats and rabbits confirmed the findings *in vitro*: uptake by the early myeloid cells, and no uptake by the nucleated red cells or lymphocytes. Repeated large doses of ^{35}S sulphate (1–2 μC/g. daily for seven days) were found to be non-toxic in animals.

In view of the localization of the isotope in the myeloid foci of the marrow and of the low energy emission of ^{35}S (0.167 MeV), a fairly localized irradiation of the myeloid cells of the bone marrow could be expected. It was decided to attempt to study the uptake of ^{35}S sulphate *in vivo* in human bone marrow, and to investigate the effect of such treatment in acute leukaemia.

Material and Method

Six patients with acute leukaemia were chosen for the study: two cases of myeloblastic-paramyeloblastic leukaemia, two cases of myeloblastic-monoblastic leukaemia, and two cases of lymphoblastic leukaemia.

The ^{35}S was obtained from the Atomic Energy Research Establishment, Harwell ($Na_2{}^{35}SO_4$, carrier free, sterile, *p*H 7, in physiological saline, 1 mC/ml. activity). The administration was by intravenous injection except in two young children, where it was given intragluteally with hyaluronidase to hasten absorption from the site of injection.

The doses ranged from 1 to 10 mC on alternate days, the total dose varying between 25 and 100 mC. The urine of the patients was collected and the activity measured.

Daily full blood counts were performed on the patients: Hb, total white blood and differential counts, and platelet counts. Apart from the two children, sternal marrow was examined both at the beginning and at the end of the ^{35}S sulphate course. Autoradiographs were prepared from the marrow and from the peripheral blood at the end of ^{35}S sulphate treatment (Lajtha, 1954).

Results

Measurements on the radioactivity of the urine indicated that over 80% of the intravenously administered ^{35}S sulphate is excreted within six hours. No subjective or objective side-effects were noted in any of the patients.

The clinical and haematological course of leukaemia was not altered in any of the patients.

The autoradiographs from the bone marrows indicated a maximum uptake of the order of 500 to 1,000 atoms ^{35}S in the early promyelocytes and myelocytes, and considerably less in the blast cells. This uptake, however, was noted only in those patients who received large doses of ^{35}S sulphate (10 mC on seven alternate days).

Discussion

The experiments demonstrated the uptake of ^{35}S sulphate by human myeloid cells *in vivo*. The two cases of lymphoblastic leukaemia (i.e., blast cells accompanied by large and small lymphocytes) were included in the series because experiments *in vitro* indicated an uptake of ^{35}S by several blast cells from the peripheral blood.

Calculations on the probable radiation dose to the cells by the incorporated ^{35}S indicated that in our series the maximum probable dose within a clump of myeloid cells may be of the order of 4 rads/24 hours. In most cells, however, the radiation dose would be considerably less. Considering the limited life span of the cells in the bone marrow, and the slow dose rate of radiation, no significant growth-inhibiting effect can be expected.

Summary

Following experiments *in vitro* which indicated an uptake of ^{35}S by human bone marrow cells, six patients with acute leukaemia were given courses of ^{35}S sulphate.

The uptake of ^{35}S sulphate by blast cells and early myeloid cells in human bone marrow *in vivo* has been demonstrated.

The ^{35}S sulphate treatment did not depress the marrow elements and did not produce improvement in the course of the disease.

This work is part of an investigation carried out under a full grant from the British Empire Cancer Campaign.

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J. clin. Path. (1955), **8**, 168.

HAEMORRHAGIC DIATHESIS DUE TO A CIRCULATING ANTICOAGULANT INVESTIGATED BY THE THROMBOPLASTIN GENERATION TEST

BY

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A circulating anticoagulant which inhibits or delays the formation of thromboplastin is now a recognized cause of acquired haemorrhagic diathesis (Biggs and Macfarlane, 1953). Reported cases of this condition may be divided into three groups. The first group consists of true haemophiliacs in whom the appearance of the anticoagulant follows blood transfusion. In the second group the anticoagulant develops shortly after a pregnancy in women who previously had shown no haemorrhagic tendency. The third group is a rather heterogeneous collection in which the anticoagulant is unassociated with haemophilia or recent pregnancy. It may develop in the absence of any other recognized disease (Joules and Macfarlane, 1938; Harrington, Desforges, Stohlman, Crow, and Moloney, 1950; Pons and Torregrosa, 1952; Hougie, 1953), but more often it is associated with some other condition, for example, syphilis and tuberculous lymphadenitis (Lozner, Jolliffe, and Taylor, 1940), non-specific lymphadenopathy (Conley, Rathbun, Morse, and Robinson, 1948), rheumatoid arthritis with amyloid deposits in lymph nodes (Collins and Ferriman, 1952), chronic nephritis and syphilis (Conley *et al.*, 1948), pemphigus treated by arsenicals (Quick and Stefanini, 1948; Dieter, Spooner, and Pohle, 1949), dermatitis (Tzanck, Soulier, and Blatrix, 1949), and myocardial infarction (Singer, Mond, Hyman, and Levy, 1950). The cases to be described belong to the third group.

Methods of Investigation

Thromboplastin Generation Test.—This test was performed as described by Biggs and Douglas (1953). Some of these tests were kindly undertaken by Dr. R. Biggs at the Radcliffe Infirmary. The remainder were performed at the Derbyshire Royal Infirmary. A number of tests were carried out in parallel at both laboratories.

The clotting times were measured by the method of Lee and White (normal 4–10 minutes), bleeding times by Duke's method (normal 2–5 minutes), and platelets by Lempert's method (normal 150,000–350,000/c.mm.), and prothrombin activity by Quick's one-stage method.

Case Summaries

Case 1.—Mrs. D., aged 45 years, a forewoman in hosiery, was well until May, 1952, when she developed severe bruising of the limbs following trivial injuries. There was no history of transfusion or recent pregnancy. She had two healthy children aged 19 and 14 years. The bruises gradually subsided on symptomatic treatment. In October, 1952, further bruising occurred, with haemorrhages into the neck and mouth causing dysphagia and trismus. Remissions and exacerbations followed. During 1953 she developed haemarthroses of both knees. Retrobulbar haemorrhage of the right eye, following a blow, resulted in blindness of this eye. In April, 1954, severe exsanguination followed massive perirenal haemorrhage. She recovered, but the bruising tendency still persists.

Investigations.—Clotting times were 4 to 50 min., usually over 30 min., and bleeding times 2½ to 7 min. Prothrombin activity was 100%. Platelets numbered 260,000–470,000/c.mm. Haemoglobin was 3.7 g.% and red cells 1,770,000/c.mm. (after haemorrhage). The marrow showed a normoblastic reaction. Liver function tests were normal. A thromboplastin generation test showed no antihaemophilic globulin and a circulating anticoagulant.

Treatment.—Transfusions of fresh blood controlled the anaemia, but did not significantly reduce the clotting time. Cortisone, 50–100 mg. daily, reduced neither the clotting time nor the titre of the anticoagulant, but appeared to check the bruising tendency.

Case 2.—Mrs. B., aged 61 years, a bus cleaner, in October, 1951, suddenly developed severe spontaneous bruising, beginning in the left arm and becoming generalized. She had no history of bruising or transfusions before. At 59 years she suffered symmetrical arthritis of the wrists, elbows, shoulders, and knees. In March, 1952, she developed severe haematuria and melaena lasting two months. In July, 1952, a spontaneous haematoma of the right eye spread to involve the face (Fig. 1). With rest and fresh blood transfusion this subsided (Fig. 2).

Investigations.—Bleeding times were 2 to 3 min. Prothrombin activity was 100%. Platelets numbered 300,000–440,000/c.mm. Haemoglobin was 6.4 g.% and red cells 2,500,000/c.mm. (during haematuria). Liver

FIG. 1.—Case 2, 11 months after the onset of disease.

FIG. 2.—Case 2, 14 months after the onset of disease.

function tests were normal. Clotting times were 15–40 min. until April, 1953. The clotting time was reduced from 33 min. to 18 min. by the addition of 1 part normal blood to 9 parts patient's blood. (A corresponding addition of normal to known haemophilic blood reduced the clotting time from 31 to 6¼ min.) A thromboplastin generation test (January, 1953) showed no antihaemophilic globulin but a circulating anticoagulant.

Treatment.—Fresh blood transfusions controlled haemorrhages but never reduced clotting times below 15 min. During cortisone therapy clotting times fell to within normal limits (Fig. 3). No relapse followed withdrawal of cortisone. In May, 1953, the anticoagulant was barely detectable. By April, 1954, it had disappeared and antihaemophilic globulin activity had returned. The patient is now well.

Experimental Results

Thromboplastin Generation Test.—The presence of a circulating anticoagulant in these patients was demonstrated using the thromboplastin generation test. In this test a mixture is made of $Al(OH)_3$-treated normal plasma, normal serum, normal platelets, and $CaCl_2$. In this mixture a very powerful thromboplastin develops. An anticoagulant which inhibits thromboplastin formation can be demonstrated by its action when added to this normal system. The approximate potency of the inhibitor can be measured by finding the highest dilution at which inhibition can be demonstrated. The method can of course also be used to demonstrate antihaemophilic globulin deficiency by replacing the normal $Al(OH)_3$ plasma by that of the patient.

Demonstration of the Inhibitor in Case 1.—Throughout the patient's illness her plasma was found to inhibit thromboplastin formation. Initially the inhibitor could only just be detected. Later it increased in potency, reaching a fluctuating maximum after about 18 months. On occasions it was detected at dilutions of the patient's plasma of over 1 in 1,000. The inhibitor's effect is clearly demonstrated in Fig. 4.

Absence of Antihaemophilic Globulin in Case 1.—At no time was any antihaemophilic globulin activity detected. Even in the early stages, when the anticoagulant was very weak, replacement of the $Al(OH)_3$-treated plasma in the "normal system" by that of the patient resulted in negligible thromboplastin formation (Fig. 5). The possibility of Factor V deficiency was excluded by the normal prothrombin time.

Ability to Correct the Clotting Defect in Haemophilia.—Initially when the anticoagulant was very weak the ability of the patient's plasma to correct the clotting defect in haemophilia was tested (Fig. 6). The patient's plasma did not correct the haemophilic abnormality.

Results with Same Tests in Case 2.—This patient differed from Case 1 in that the anticoagulant finally disappeared. During the active phase of the disease the anticoagulant was clearly demonstrated (Fig. 7). After 18 months the patient was clinically improved and little anticoagulant activity could be

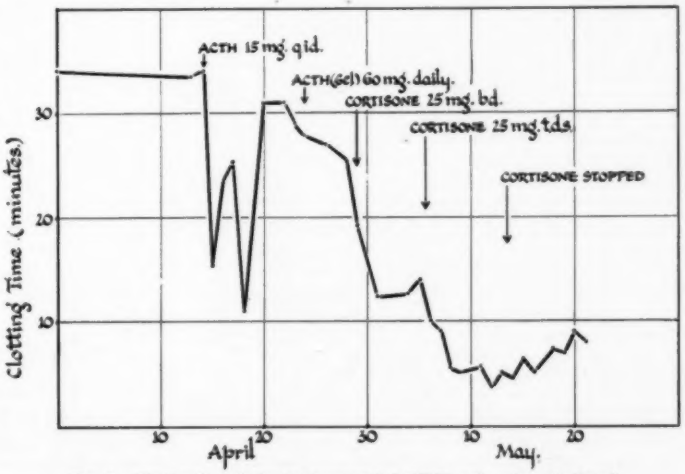

FIG. 3.—Clotting times during treatment with A.C.T.H. and cortisone in Case 2.

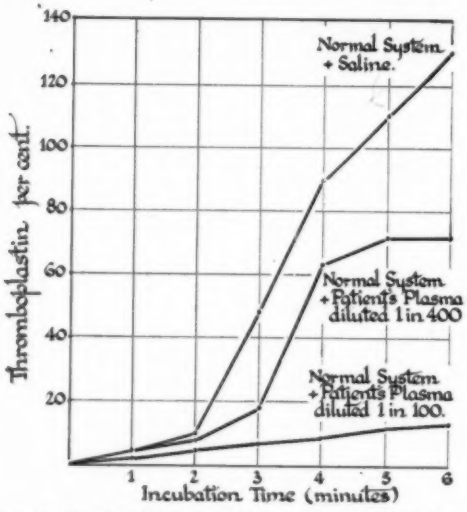
Normal System + Saline.
Normal System + Patient's Plasma diluted 1 in 400
Normal System + Patient's Plasma diluted 1 in 100.
Thromboplastin per cent.
Incubation Time (minutes)

FIG. 4.—Thromboplastin generation test demonstrating inhibitor in Case 1 18 months after the onset of disease. "Normal system"=normal serum, normal platelets, and normal $Al(OH)_3$ plasma, with $CaCl_2$ which initiates incubation; 1 vol. 0.85% NaCl added to the control "normal system"; 1 vol. diluted patient's plasma added to test the "normal systems." Dilutions are final dilutions in incubation mixture. Thromboplastin formation inhibited by high dilutions of patient's plasma.

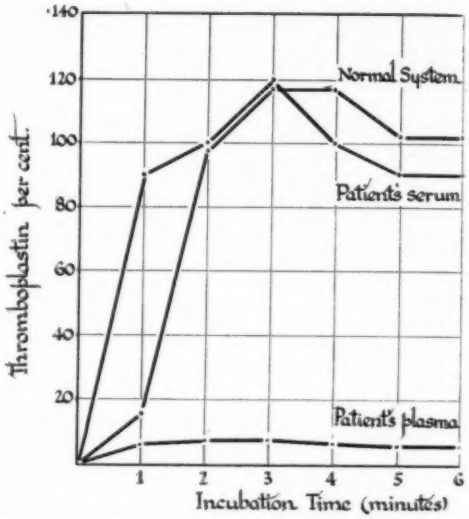

FIG. 5.—Thromboplastin generation test showing absence of anti-haemophilic globulin activity in Case 1 five months after the onset of disease when the anticoagulant was very weak. "Patient's serum"="normal system" with serum replaced by patient's serum. "Patient's plasma"="normal system" with $Al(OH)_3$ plasma replaced by patient's $Al(OH)_3$ plasma. Thromboplastin formation was negligible with the latter.

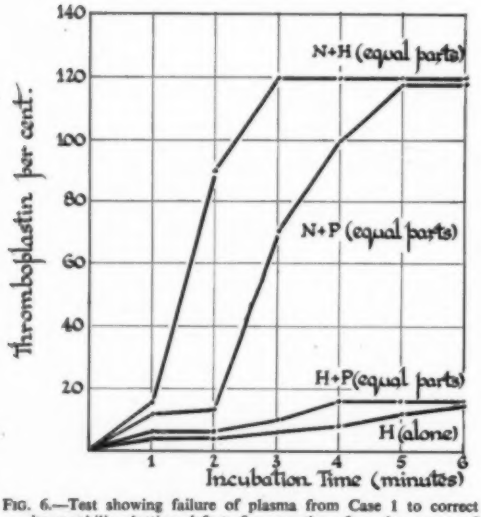

FIG. 6.—Test showing failure of plasma from Case 1 to correct haemophilic clotting defect five months after the onset of disease. N=normal $Al(OH)_3$ plasma. P=patient's $Al(OH)_3$ plasma. H=haemophilic $Al(OH)_3$ plasma. Mixtures as indicated replaced $Al(OH)_3$ plasma in "normal system." The haemophilic defect was not corrected by patient's plasma.

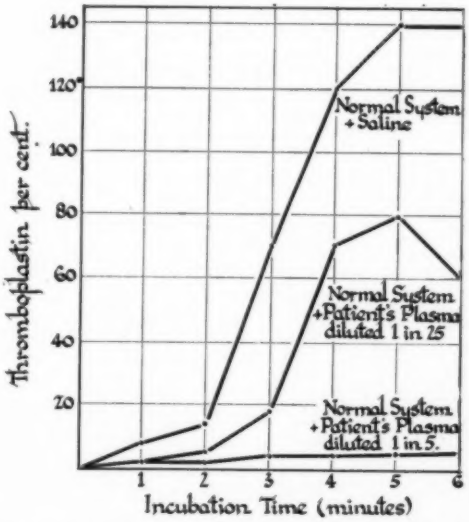
Normal System + Saline
Normal System +Patient's Plasma diluted 1 in 25
Normal System +Patient's Plasma diluted 1 in 5.
Thromboplastin per cent.
Incubation Time (minutes)

FIG. 7.—Test demonstrating inhibitor in Case 2 14 months after the onset of disease. Dilutions of patient's plasma are final dilutions in incubation mixture.

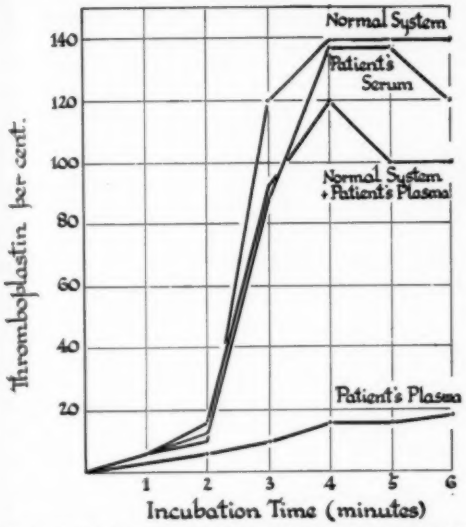

FIG. 8.—Test during recovery of Case 2 showing deficiency of antihaemophilic globulin but little inhibitory effect 18 months after the onset of disease. "Patient's serum" = "normal system" with serum replaced by patient's serum. "Patient's plasma" = "normal system" with $Al(OH)_3$ plasma replaced by patient's $Al(OH)_3$ plasma, thromboplastin formation being negligible. "Normal system" + patient's plasma = patient's $Al(OH)_3$ plasma added to normal system, thromboplastin formation being practically normal.

detected. At this time it is of note that, although little inhibitory effect could be detected, the patient's plasma still showed a deficiency of antihaemophilic globulin (Fig. 8). A year later the antihaemophilic globulin had returned to normal.

Discussion

Both cases present a clinical picture which resembles haemophilia starting in middle life. Haemarthroses occurred, but haemorrhage into the soft tissues was a more prominent feature. As in haemophilia the clotting time was prolonged and the bleeding time was normal. This resemblance to haemophilia is in keeping with the absence of antihaemophilic globulin activity shown by the thromboplastin generation test, further confirmed by the failure of the patient's blood to correct the clotting defect of a known haemophiliac. In addition a circulating anticoagulant was clearly demonstrated in both cases.

There was an approximate correlation between the patient's clinical condition and the titre of the anticoagulant. In both cases acute haemorrhagic episodes were associated with high titres of anticoagulant. The recovery phase of Case 2 was associated with diminution and finally the disappearance of the anticoagulant. It must, however, be mentioned that Case 1 appeared clinically well for several weeks when the circulating anticoagulant was demonstrated at titres of over 1 in 400. It is not clear whether this apparent temporary immunity to the circulating anticoagulant was related to the coincident cortisone therapy. Nor is it certain that the disappearance of the anticoagulant in Case 2 was due to the cortisone given at that time.

The thromboplastin generation test findings indicate that the anticoagulant acts by inhibiting thromboplastin formation. The absence of antihaemophilic globulin activity in both cases suggests that the site of action of the anticoagulant is on the antihaemophilic globulin itself.

In Case 2 the antihaemophilic globulin activity returned following the disappearance of the circulating anticoagulant. This is what would be expected if the anticoagulant specifically inactivated antihaemophilic globulin.

Craddock and Lawrence (1947) presented evidence that the anticoagulant which develops in some haemophiliacs is an immune antibody whose corresponding antigen is the antihaemophilic globulin of transfused blood. This has been confirmed by Frommeyer, Epstein, and Taylor (1950). It has also been suggested that the anticoagulant in both haemophiliacs and non-haemophiliacs may be of a similar nature (Quick and Stefanini, 1948; Collins and Ferriman, 1952). This would imply that the anticoagulant developing apparently spontaneously in non-haemophiliacs may be auto-antibody. By analogy with the auto-antibody of acquired haemolytic anaemia a favourable response of at least some cases to cortisone and A.C.T.H. is not a surprising finding.

Summary

Two cases are presented of severe acquired haemorrhagic diathesis occurring in women unassociated with haemophilia or recent pregnancy.

They resemble haemophilia clinically and show a prolonged clotting time with a normal bleeding time.

The thromboplastin generation test revealed an anticoagulant which inhibited thromboplastin formation. It also demonstrated the absence of antihaemophilic globulin activity.

The absence of antihaemophilic globulin activity was related to the presence of the anticoagulant. In the second case disappearance of the anticoagulant coincided with cortisone therapy, after which antihaemophilic globulin activity returned.

I wish to thank Dr. R. Biggs for her guidance and many practical suggestions. I am indebted to Dr. D. V. Hubble and Dr. G. E. Kidman for permission to publish details of their cases, and to Dr. H. Barber and Dr. G. R. Osborn for their encouragement. I should also like to thank Mr. J. W. Allen for his assistance in carrying out the tests, and Mr. R. Foster for the graphs.

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THE EFFECT OF ANTIBIOTICS ON THROMBOPLASTIN GENERATION

BY

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(RECEIVED FOR PUBLICATION SEPTEMBER 15, 1954)

The effect of antibiotics on the blood coagulation mechanism has attracted the attention of many workers in various countries. Investigations have mostly been based on changes in the clotting time before, during, and after administration of the drugs *in vivo* (Parker and Wright, 1952; Galt and Hunter, 1950; Miller and Bass, 1952; Casassa, 1952; Macht, 1952; and many others). This approach has led to confusing and conflicting reports, and to the indictment on these grounds of almost every antibiotic at various times. Florey and Jennings (1949) remarked that

> "In view of the difficulty of making reliable and consistent estimates of coagulation time . . . it is justifiable to accept reports of abnormality with reserve until they are fully confirmed, especially as little or nothing to bear them out has been reported from among the thousands of patients treated in the clinic."

In addition to the effect on the clotting time, a few reports have appeared in which the effect on other tests has been studied (e.g., heparin titration and one-stage prothrombin times). No attempt appears to have been made otherwise to localize the site of action, resulting in an observed clotting time change, within the coagulation mechanism. The difficulty in interpreting the findings in the literature caused us to attempt a fresh approach to the problem by means of newer coagulation techniques. The possibility of thrombosis or haemorrhage due to, or aggravated by, these drugs may have a bearing on their clinical use; our interest has been especially in connexion with the effect that they might have in patients already suffering from a haemorrhagic condition, e.g., acute leukaemia with thrombocytopenia, haemophilia, etc.

Methods

A modified Lee-White clotting time technique was used with siliconized glass tubes. Venous blood, collected with as little trauma as possible, was placed in 1 ml. quantities in three clean, dry, siliconized tubes, 75×8 mm. at 37° C. Beginning after three minutes, the first tube was tilted at 30-second intervals until clotting occurred. The second tube was then tilted at the same intervals until it, too, clotted; finally, the third tube was similarly tilted, and its time recorded as the clotting time for the test in question. With this method, our normal range is 13–18 minutes. Siliconized tubes were used to magnify any changes which might occur in the clotting time, and also to eliminate a foreign surface effect as far as possible. In the case of haemophilic blood, non-siliconized tubes were used, because of the length of time otherwise involved. For testing of an antibiotic *in vitro*, 0.1 ml. of the desired concentration of the drug in 0.85% saline was placed in each of one set of tubes, and 0.1 ml. of saline alone in another set as control; the blood was gently agitated with both solutions before being allowed to clot. This probably explains why our normal (silicone) clotting times are somewhat shorter than those generally reported in the literature.

The one-stage prothrombin time method of Quick was used to exclude changes in the prothrombin complex.

Prothrombin consumption was measured by obtaining the serum one-stage prothrombin time one hour after clotting had occurred (the clotting time test-tubes could be used for this). Human fibrinogen at a concentration of 7 to 9 mg./ml. in borate buffer was employed, and the minimal time for normal blood was 25 seconds (30 seconds when non-siliconized glass is used).

The thromboplastin generation test of Biggs and Douglas (1953) was modified by the use of a brain extract, as previously described by Bell and Alton (1954), in place of the platelet suspension. By the use of this extract, and with two workers, we have found our normal "peak" times to be 10 ± 1 seconds, with an accuracy of ± 0.5 second. Ten seconds was arbitrarily chosen as representing 100% thromboplastic activity. To test the effect *in vitro* of a given antibiotic on thromboplastin generation, 0.3 ml. of 0.025 M. calcium chloride solution was added to an incubation mixture containing in each case a 1 in 100 dilution of the brain extract, a 1 in 5 dilution of normal "alumina plasma," a 1 in 10 dilution of normal serum, and the required concentration of the selected antibiotic. At 1-minute intervals 0.1 ml. of this incubation mixture and 0.1 ml. of the $CaCl_2$ solution were simultaneously added to 0.1 ml. volumes

of normal plasma at 37° C., and the clotting times noted. All results were read from the same dilution curve in terms of percentage thromboplastic activity of the saline control which was run before and after each set of tests.

Results

In Vitro.—Fig. 1 shows the results obtained when various antibiotics were added to the thromboplastin generation incubation mixture in varying concentrations; these were selected to cover adequately the usual therapeutic levels used clinically. From these results, it would appear that oxytetracycline (terramycin) may augment thromboplastin generation slightly, penicillin has no significant effect, whereas the other antibiotics (especially aureomycin) appear to decrease thromboplastin generation to a greater or less extent. These effects appear with concentrations falling within the range of therapeutic levels. Although not shown, magnamycin produced no significant effect.

It was found that the effect of 0.1 mg./ml. of aureomycin could be partly reversed by increasing the concentration of the alumina plasma five times (i.e., using it undiluted), and completely reversed by increasing that of the serum 10 times (undiluted). Therefore it could be postulated that the drug was decreasing thromboplastin generation by rendering ineffective some factor present in serum, and also in alumina plasma to a lesser extent, at a critical level. One known factor which would seem to fulfil these criteria is the Christmas factor (P.T.C.).

In Vivo.—The results of administering four antibiotics to three normal controls are shown in Fig. 2; the same normal subjects were used for each study. The tests were carried out in the non-fasting state, as an approximation to ordinary clinical conditions; however, in some cases, the experiment was repeated in the fasting state, with much the same results. All tests were performed immediately before, and three and a half hours after, administration of the drug. In general, these results supported those of the tests *in vitro*. To be considered significant, all the normals had to show the same trend to a greater or lesser extent. The lack of significant change in the one-stage prothrombin time was considered as evidence that no appreciable change

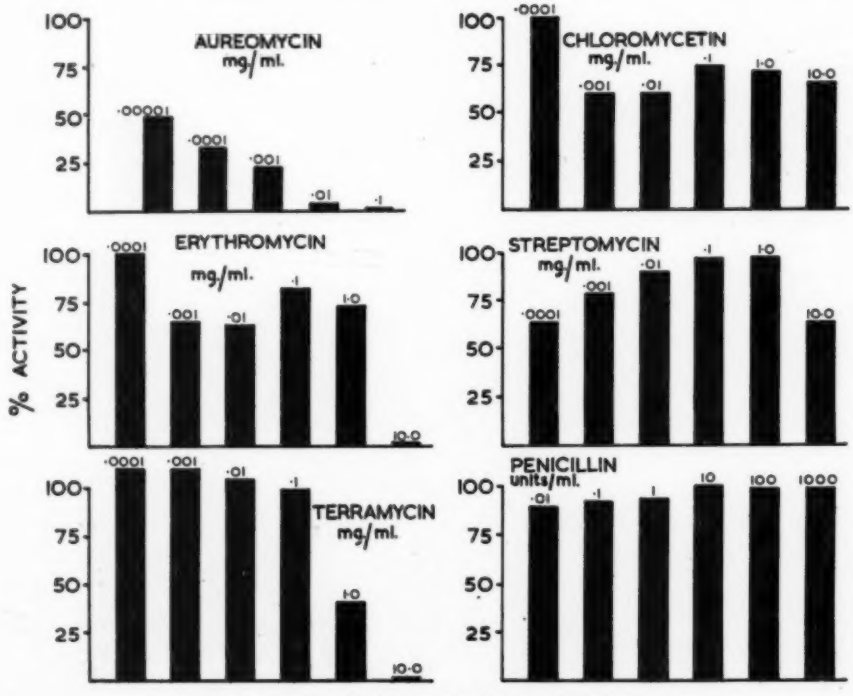

FIG. 1.—Effect *in vitro* of antibiotics in varying dose on thromboplastin generation.

was occurring in the prothrombin, Factor V, Factor VII, or fibrinogen levels. The clotting time varied directly with the prothrombin consumption, suggesting that the site of action was at the level of formation of thromboplastin.

An attempt was made to throw more light on the factor involved by studying the changes in the thromboplastin generation test. When 1 in 10 dilutions of pre- and post-administration sera were compared, no significant difference could be noted. When the undiluted sera were used, the post-administration serum showed a significant decrease in activity (Fig. 3). A 50% dilution of this serum with normal undiluted serum caused complete correction, as did undiluted haemophilic serum (which corrected even more than did the normal). However, a 50% mixture of post-administration serum and Christmas disease serum did not correct the defect. This finding served to strengthen

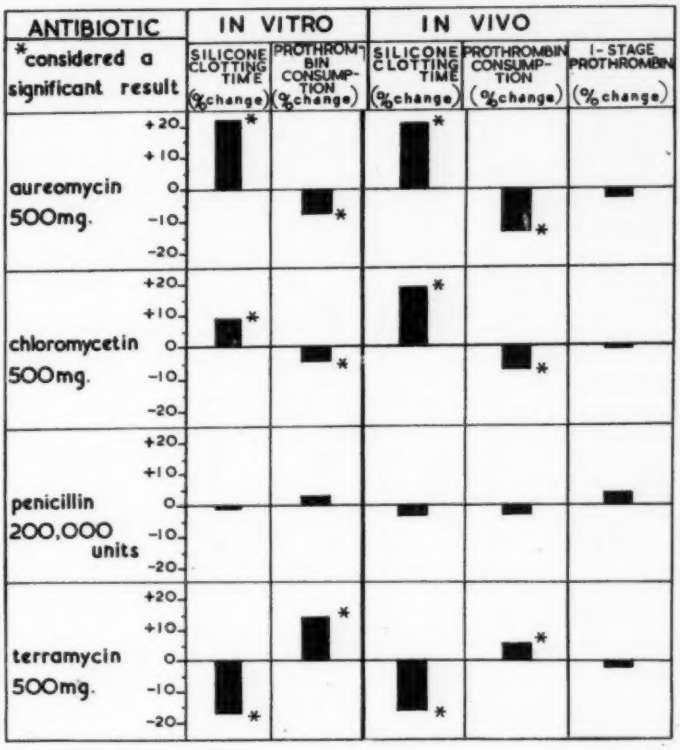

FIG. 2.—Effects *in vitro* and *in vivo* of various antibiotics on the normal coagulation mechanism.

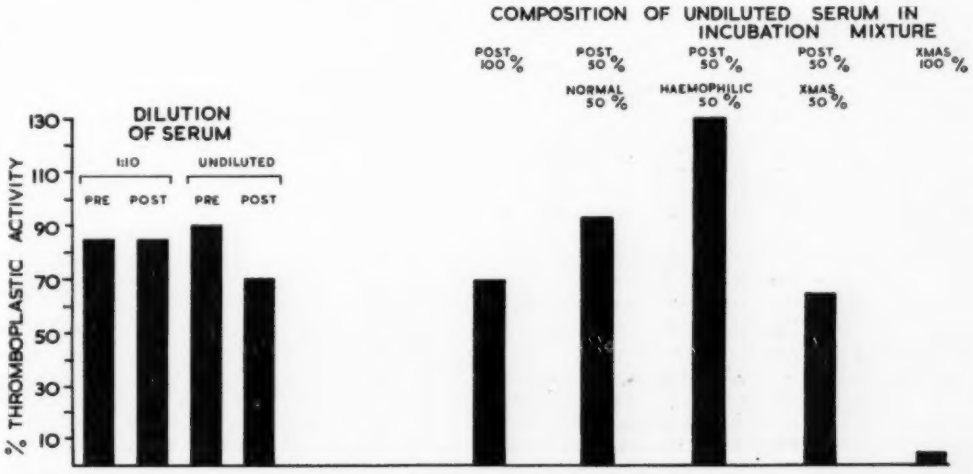

FIG. 3.—Defect in undiluted serum four hours after ingestion of 500 mg. aureomycin and its correction by various sera.

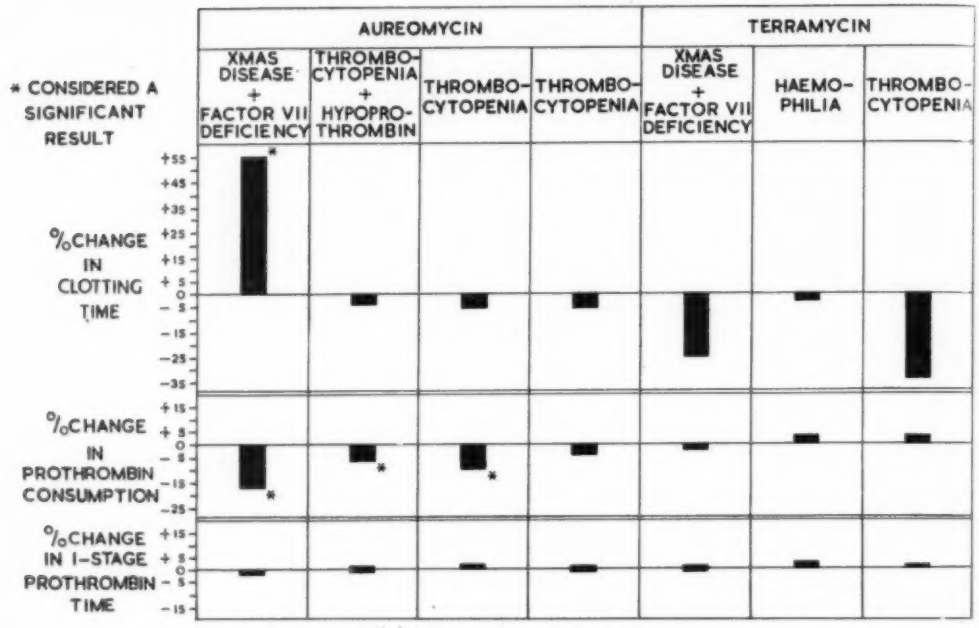

FIG. 4.—Effect of aureomycin and terramycin *in vivo* in bleeding states.

our conjecture that aureomycin may act by reducing the effective level of Christmas factor in the blood. Similar results, to a less marked extent, were found with post-administration serum in the case of chloramphenicol (chloromycetin).

Finally, because of our interest in the effect of these drugs on patients with haemorrhagic disorders, we gave 500 mg. of aureomycin and of terramycin to patients with thrombocytopenia, haemophilia, and one with Christmas disease combined with Factor VII deficiency (Fig. 4). Terramycin had no significant effect in any of these patients, and the slight increase in thromboplastin generation noted in the normals could not be demonstrated. Aureomycin again produced a decrease in thromboplastic activity, as judged by the prothrombin consumption test. Of particular interest is the effect in the patient with the combined Factor VII and Christmas deficiency. The unchanged one-stage prothrombin time and Factor VII determination showed that aureomycin apparently does not affect Factor VII. The significant aggravation of his Christmas disease state is a further piece of evidence in favour of the suggestion that it is by way of the Christmas factor that aureomycin affects the coagulation mechanism.

Discussion

Biggs, Douglas, and Macfarlane (1953) have shown that the components necessary for the generation of a potent plasma thromboplastin are a platelet factor (or factors), anti-haemophilic globulin, Factor V, Factor VII, and Christmas factor. Of these, Factor VII and the Christmas factor are predominantly serum factors, although the latter also exists in plasma. Platelet numbers and function were not studied in this investigation, but previous studies by one of us (W. N. B.) suggest that a platelet factor is not the principal site of action of aureomycin. Reversal of the aureomycin effect by increasing the concentration of platelet-free alumina plasma or platelet-free serum also suggests that we are concerned rather with a substance in serum, and to a minor extent in alumina plasma, than with a platelet factor. The constancy of the one-stage prothrombin time, both in the normal and in a patient with a Factor VII deficiency, demonstrates that Factor VII is not the responsible serum component. The correction of the aureomycin post-administration thromboplastin generation curve with sera other than that of Christmas disease, and the accentuation of the abnormal tests in a patient with Christmas disease, suggests that

this component is primarily involved in the action of aureomycin; the effect is apparently exerted by inhibition of the Christmas factor, since increasing the concentration of the plasma or serum abolishes it to a varying degree. No such acquired deficiency of Christmas factor has been reported to date.

The results with terramycin are difficult to interpret. Although the tendency seems to be slightly towards hypercoagulability in the normal, no significant effect occurs in the presence of hypothromboplastinaemia. Were the former significant, it would be expected that a clinical predisposition to thrombosis would have been reported by this time; we are not aware of any such report up to the present.

Though technically significant, these studies do not indicate any serious impairment of the normal coagulation mechanism by any of the drugs considered. This is not the case, however, where there is already a defect in the generation of plasma thromboplastin due to a deficiency of Christmas factor. In other hypothromboplastinaemic states, the position appears to be more akin to that in the normal subject. A therapeutic dilemma from this cause should therefore be quite rare, save for the specialist in coagulation problems.

There are obviously many aspects of this question which require fuller investigation, such as the relation of these findings to the route of administration, duration of administration, and size of dose. This preliminary report is meant simply as a suggestive guide to a more promising technical approach than is offered by the simpler tests alone.

Summary

During study *in vitro* and *in vivo* of the effect of several antibiotics on blood coagulation, no evidence of hypercoagulability was found, except with terramycin, and its effect was doubtfully significant. Although other minor effects were observed on the coagulation mechanism of normal subjects, it was not felt that these had any clinical implications.

No significant change occurred in the prothrombin complex with any of the antibiotics studied. Aureomycin, and to a minor degree chloromycetin and probably erythromycin, impaired thromboplastin generation, apparently by reducing the effective level of circulating Christmas factor. Terramycin and penicillin had no deleterious effect on thromboplastin generation.

Preliminary studies, in hypothromboplastinaemic conditions, suggest that aureomycin and chloromycetin may significantly aggravate the defect of Christmas disease.

We are grateful to Sir Lionel Whitby, Regius Professor of Physic, and to Dr. F. G. J. Hayhoe for providing facilities and encouragement for this work, which was supported by the Elisabeth Storey Fund. Invaluable technical help was given by Miss S. C. Tomlin, and Messrs. B. W. Gurner and R. J. Flemans prepared the graphs for publication.

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ASSOCIATION OF CLINICAL PATHOLOGISTS: 54th GENERAL MEETING

The 54th general meeting was held in Douglas, Isle of Man, from April 13 to 15. Twenty-four papers were read and a morning session was devoted to demonstrations. Summaries of some of the papers are printed below.

Lower Nephron Nephrosis with Little or No Oliguria

S. SEVITT (Birmingham Accident Hospital) showed that distal tubular necrosis (lower nephron nephrosis) in the kidneys of burned patients occurred more frequently among those who had an adequate or normal flow of urine than it did associated with severe oliguria or anuria. Clinically and biochemically uraemic and non-uraemic forms with little or no oliguria occur. The uraemic group is important to diagnose because the recent advances in therapy may reduce its high fatality rate. Morphologically the kidneys of this group showed a diffuse distal tubular necrosis in which haemoglobin casts were prominent. Many nephrons are involved, and although the morphology was no different from that found in those with anuria or severe oliguria, tubular function is less impaired. The finding of non-oliguric uraemia in an injured man with histological distal tubular necrosis of the kidneys indicated that the phenomenon was not restricted to burned subjects.

In the non-uraemic condition significant azotaemia did not occur during the course of the illness and death was due to non-renal complications. Histologically the kidneys of these patients should either be a focal type of distal tubular necrosis associated with few casts and affecting only a minority of the nephrons, or the healing or healed evidence of a recent focal attack.

Observations on the Sensitivity of the Haemolytic System in Complement-fixation Tests

H. M. RICE (General Hospital, Nottingham) said that sera giving weakly positive results with the Price (Whitechapel) Wassermann technique in the morning screen test were not infrequently found negative when the quantitative test was performed in the afternoon, using the same haemolytic system.

Parallel tests, in triplicate, were therefore made using new (freshly prepared), old (the morning suspension after standing at room-temperature for five to six hours), and split suspensions (part of the morning suspension, taken immediately after adding the haemolysin, kept at 4° C. and sensitized at 37° C. immediately before use). Two hundred and fifty sera, from unselected, consecutive routine tests on hospital and clinic patients, were examined in triplicate. Sera insufficient for all three were tested with new and old suspensions only, such additional sera making the N/O series up to 400.

A difference of 100% is not significant in doubling-dilution techniques. Nevertheless, the numbers encountered showed marked differences, tending to support the evidence of the higher readings.

New exceeded old—67 (×2), 10 (×4), 5 (×8), and 2 (×16)	total 84
Old exceeded new—28 (×2)	,, 28
New exceeded split—58 (×2), 12 (×4), 4 (×8), and 1 (×16)	,, 75
Split exceeded new—33 (×2)	,, 33

The differences between split and old cells were very much less.

Split exceeded old—48 (×2) and 9 (×4)	total 57
Old exceeded split—45 (×2) and 3 (×4)	,, 48

Both old and split cells were thus more readily lysed than new.

The full number (400) of the N/O series showed similar results.

New exceeded old—115 (×2), 14 (×4), 7 (×8), and 2 (×16)	total 138
Old exceeded new—33 (×2)	,, 33

Complement titrations repeated with old cells showed a tendency to " tailing-off," rather than a clear-cut end-point, confirming the increased tendency to lysis of old cells.

No evidence was found, in a series of 50 parallel tests, that agitation during the period of sensitization affects the sensitivity of the haemolytic system.

It is concluded that, where strict accuracy of quantitative results is required in complement-fixation tests, it is essential that the haemolytic system be freshly prepared.

Human Haemoglobin Variants

H. LEHMANN (St. Bartholomew's Hospital, London) said that under human haemoglobin variants are understood forms of haemoglobin which have the same haem but which differ in the nature of their globin. The two normal haemoglobin variants of man are the foetal and the adult forms (F and A). The F variant is prevalent during intrauterine life and disappears during early infancy; if an anaemia such as sickle-cell anaemia or thalassaemia major begins its course during early infancy F may persist into adult life. Thus F is of no pathological significance by itself and usually only indicates that an anaemia is present which started in early infancy. There are patients with sickle-cell anaemia and with thalassaemia major in whom no or nearly no F is present. There is reason to believe that F persists longer in child populations with anaemias due to malnutrition than in healthy children.

Unlike F, A, and all the other known haemoglobin variants (except possibly one recently briefly described as H) (Rigas, Koler, and Osgood, 1955) are inherited on

simple Mendelian principles: one gene from each parent. There are known the variants S (sickle), C, D, E, and G. In thalassaemia no abnormal haemoglobin is found, but the A gene is altered (A^T) in such a way as to lower the haemoglobin A synthesis; the homozygote for the altered A gene (A^TA^T) shows a hypochromic anaemia without iron deficiency, i.e., without depletion of iron stores in the bone marrow (A, S, C, D, E, F, and G are internationally recognized symbols (*Blood*, 1953), but the author used A^T only to denote the faulty production of A in thalassaemia). Variant F is differentiated from A by its resistance to alkali. All the haemoglobin variants, except S and D, can be differentiated by electrophoresis, though an AF mixture shows only poor resolution. Variants S and D are identical on electrophoresis, but S differs from all other haemoglobins by the low solubility of its reduced form. This low solubility is of course the cause of the intracellular gelling of sickle-cell haemoglobin when the cells are reduced with the result of "sickle-cell" formation.

Most people are homozygous for A (AA), but if A is inherited from one parent and an abnormal haemoglobin from another, heterozygotes result. These are called "carriers" of abnormal haemoglobins. As long as one of the two genes is a normal A usually no serious anaemia or disease occurs. An A A^T individual is said to show thalassaemia minor: AS, AC, AD, AE, and AG individuals are said to carry the sickle-cell trait, the haemoglobin C trait, the haemoglobin D trait, and so forth. If both A genes are replaced by a variant, anaemia will result, though this will not invariably be so. A^TA^T is the combination found in thalassaemia major (Neel and Valentine, 1947). SS is the combination in classical sickle-cell anaemia found in the family studies of Neel (1949), and verified by the first electrophoretic analysis by Pauling, Itano, Singer, and Wells (1949). The combinations SA^T, SC, and SD are also known and they usually give rise to a modified sickle-cell anaemia. Other combinations in which no A is present and which result in minor haemolytic anaemias are CA^T, CC, EA^T, EE. The only case of GG seen so far was not anaemic, though a measurement of red cell survival could not be made and it is possible that a mild anaemia was compensated by increased blood production.

KNOWN COMBINATIONS OF HAEMOGLOBIN VARIANTS

AA		Normal adult
AA	F	Infants
AA^T	F (not always present)	Thalassaemia minor
A^TA^T	F (nearly " ")	" major
AS		Sickle-cell trait
SS	F " " "	" " disease
SA^T	F (presence of A often assumed only)	Microdrepanocytic disease
AC		Haemoglobin C trait
CC	F (sometimes present)	" C disease
SC	F (not always present)	Sickle-cell haemoglobin C disease
AD		Haemoglobin D trait
SD		Sickle-cell haemoglobin D disease
AE		Haemoglobin E trait
EE		" E disease
EA^T	F (reported as EF, no A found)	Thalassaemia haemoglobin E disease
AG		Haemoglobin G trait
GG		One case, no anaemia noticed

Distribution.—The distribution of the variants shows S all over tropical Africa and finding its way into the southern, northern, and eastern coasts of the Mediterranean Sea; other enclaves are found in South Arabia and India. A^T is more frequent than S in the Mediterranean region but is absent in tropical Africa. Towards the east it extends into Asia minor, Persia, north India, and further into Asia. It is found in Siam, and, although no reports from China itself have yet been forthcoming, thalassaemia has been seen in Chinese who were resident in the West Indies.

Haemoglobin C seems to be restricted to West Africa and has not yet been seen in East Africa; some instances have been reported from North and South Africa, and it was of course discovered and studied most in the U.S.A., all areas where the gene can have been imported with West African slaves. Haemoglobin E has been seen in natives of India, Ceylon, Siam, and Indonesia, and it seems to be characteristic for south-east Asia. Haemoglobin G has so far been found in West Africa only.

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The Value of Typing in Epidemiological Investigations

W. H. BRADLEY said that roughly a quarter of a century had passed since typing began to influence epidemiological thought. This seemed to be a suitable period for an assessment to be made. Griffith had first introduced serological typing of β haemolytic streptococci in 1926 and by 1934 a range of 27 reference types was available. Craigee and Felix had shown that bacteriophage typing of *S. typhi* was practicable in 1938, and by 1947 had prepared a couple of dozen reference "phages."

Evaluation of the results was of necessity complicated. So many other things were happening at about the same time. However, regarding streptococcal disease there was a significant fall in mortality between 1934 and the advent of sulphonamides, and this fall probably reflected the considerably increased possibility of controlling outbreaks stimulated by the Griffith's typing. This probability was particularly well illustrated in the fall of deaths from puerperal sepsis.

Regarding enteric fever, the mortality in the United Kingdom had fallen more rapidly than in the other European countries. This improvement first appeared when phage typing was introduced into epidemiological practices, and the striking gain over the past 12 years was very probably related to the better epidemiological services in this country. Other applications of typing were reviewed, the investigation of salmonellosis caused by spray-dried egg during the war being one striking example.

In summary, Dr. Bradley thought that because typing made epidemiological investigations more convincing their quality was improved and preventive medicine more effectively practised. But perhaps the most important gain from typing had been in other fields. Epidemiological and aetiological concepts had been reshaped. The role of Type 12 and to a lesser extent Type 4 streptococci in the causation of nephritis was an example. It was now realized that the many distinct serological or phage types within a species were acting independently and that their specificity was enduring. Dr. Bradley was consoled by the stability revealed by typing, but he thought typing was most important because it was in line with the great strategy, divide and conquer.

The Bacteriophage Typing of *Staphylococcus aureus*

R. E. O. WILLIAMS (Staphylococcus Reference Laboratory, Public Health Laboratory Service, Colindale) said that the bacteriophages used for typing staphylococci were isolated from a number of different strains of staphylococci and had been selected to yield a set that would, between them, act on the greatest number of strains. Twenty phages were at present in use as a "basic set" and a further 19 phages held in reserve for testing otherwise untypable strains.

About 66% of coagulase-positive staphylococci are lysed by one or more of the typing phages when the phages are used at the normal working dilution; by using more concentrated preparations of phage, about 90% of strains can be identified.

Staphylococci are only rarely lysed by a single one of the 20 phages; more often two or more, up to six or eight phages, act together to produce a "pattern" of lysis. Too many different patterns are found for it to be practicable to label them as "types," so a list of the phages acting on the staphylococcus is reported as its "lytic pattern." Ordinarily, before two staphylococci are regarded as having different origins one has to be lysed strongly by two phages that are quite inactive on the second.

Four broad groups of phage patterns are recognized; three of the groups are approximately equally distributed among healthy carriers of staphylococci and in most minor septic lesions. Strains resistant to the newer antibiotics are largely confined to one group (III), which also contains practically all the strains causing food-poisoning.

The Enhancement of the Antigenic Activity of Toxoids by Sympathomimetic Drugs

J. UNGAR (Glaxo Laboratories, Ltd., Greenford, Middx.), said that water-insoluble mineral carriers such as aluminium or calcium salts, tapioca, oils and fats, and, recently, bacterial suspensions had been used to increase the efficiency of prophylactic toxoids and thereby reduce the number of injections required. Their presence causes local irritation, which is most severe with aluminium and calcium salts and least severe with bacterial suspensions; he had used *H. pertussis* vaccines successfully in the past.

As the use of vaccines might be undesirable in hypersensitive persons, he had tried to improve the antigenic efficiency of diphtheria toxoid by altering the rate of absorption with drugs exerting a local tissue effect. Adrenaline hydrochloride 1 in 1,000 and 1 in 2,000, benzedrine hydrochloride 1 in 250, ephedrine hydrochloride 1 in 250, and histamine 0.5 μg./ml. had been used. Two amines, β-phenylethylamine and phenylethylamine acetate, which have no vasoconstrictor action, were included as controls. When tested in guinea-pigs according to T.S.A. regulations the sympathomimetic vasoconstrictors caused marked enhancement of the response to diphtheria toxoid. Combination with histamine caused only slight enhancement and the two control amines did not affect the response to the toxoid.

Adrenaline hydrochloride is unstable at neutrality whereas benzedrine and ephedrine hydrochlorides are stable and can be stored in diphtheria toxoid. Vasoconstrictors may prove useful for enhancing the efficiency of diphtheria toxoid, especially when children hypersensitive to injections of antigen are being immunized.

Pneumothorax and Interstitial Emphysema in the Newborn

JOHN L. EMERY (Sheffield) reported that, in a consecutive series of 100 infants dying within seven days of birth, seven of 52 full-term children had died with interstitial emphysema. After congenital deformities it was the commonest single cause of death. The condition was spontaneous, not due to artificial respiration, but probably due to the expulsive efforts of crying in lusty infants who had inhaled vernix or mucus.

Fibrinous Vasculosis

A. C. LENDRUM (Dundee) said that this term was suggested for the deposition in blood vessel walls of a material giving the staining reactions of fibrin. The commonest cause was severe stagnant ischaemia as in infarction or torsion. Other causes were drug-induced hyperergic arteritis, extreme hypertension, polyarteritis nodosa, and occasionally acute rheumatic disease, disseminated lupus erythematosus, dermatomyositis, etc.

The fibrin is commonly seen under an intact endothelium, sometimes as a complete ring round the intima. From this level it either seeps into the wall (vessels of infarcted zone), or is injected across the media (in extreme hypertension) to spread out along the external elastica, through which it may erupt focally. At sites where a layer is traversed, there is in all types of fibrinous vasculosis a stretching and distortion of the nuclei as if they had been subjected to mechanical extension. Gross cellular aggregation is seen in and around a few, some, or all of the affected vessels. Intravascular thrombosis is uncommon except in small vessels, even when the vessel wall is a mass of fibrin.

The most extreme degree of fibrinous vasculosis had been seen in the pulmonary vessels of two cases of mitral stenosis, the next degree in infarcted organs. The three forms of fibrinous vasculosis, the stagnant, hypertensive, and hyperergic, appear to be histologically indistinguishable. All may be regarded as a form of exudation due to an upset between the pressure in the vessel and the permeability of the lining.

Incidence and Location of Lung Carcinoma in Haematite Miners

J. STEVEN FAULDS (Cumberland Infirmary, Carlisle) reported that the incidence of primary cancer of the lung in iron-ore miners in Cumberland over the last 23 years was 9.4%, which is four times that found in a control group of males of similar age and habits differing only in occupation.

The site of the tumours corresponds with the areas where iron-ore miners develop the characteristic massive fibrosis, and the primary lesion is not in the main bronchus but in a peripheral lung field. The observed association of squamous-celled lining to a chronic abscess cavity and a squamous carcinoma in the same area suggests that the tumour arises in the sidero-silicotic mass.

Since silicosis is a frequent finding in other varieties of mining and an association between silicosis and bronchial cancer not commonly seen, it is difficult to give a reason for the increased frequency of carcinoma found in iron-ore miners.

There was only one case of active pulmonary tuberculosis found in the 17 cases of carcinoma, while the incidence of active tuberculosis in the 180 iron-ore miners was 44%.

Radiographic Examination of Lung Specimens by Soft *X* Rays

GEORGE J. CUNNINGHAM (St. Bartholomew's Hospital, London) briefly referred to the non-medical use of soft *x* rays. A technique for the examination of thick lung slices was described, including the production of three-dimensional radiographs. A number of different pathological conditions were investigated and the radiographs compared with histological sections from the same slice of tissue. Serial slices of lung could be screened for the presence of localized areas of consolidation, and dilated bronchi were well shown. Excellent detail of lung structure could be obtained in such conditions as emphysema, and the distribution of the bullae was readily identifiable. Normal lungs proved much more difficult owing to collapse on slicing. Radiographic detail was naturally much better with larger amounts of residual air in the lung.

A group of pneumoconiotic lungs from coal-miners had been examined, and breaking-down carbon nodules were found to contain radio-opaque material, which, on histochemical examination, appeared to be mostly calcium with a smaller amount of iron.

It was concluded that the method was useful for teaching purposes; for obtaining accurate information on the exact distribution of certain lesions; for correlating the morbid anatomical and histological appearances.

Blood Ammonia Levels in Liver Disease

A. G. RIDDELL (Department of Surgery, University of Manchester) said that experiments on dogs with porta-caval anastomoses had shown that a neurological disorder could be produced in these animals by intoxication with ammonia. Histological examination of the brains of these dogs showed the same change in the protoplasmic astrocytes as occurs in patients dying in hepatic coma. In hepatic coma a raised blood ammonia level is found. The relationship between the neurological symptoms and the raised blood ammonia was discussed. It was concluded that the type of hepatic coma that followed bleeding from oesophageal varices was analogous to the syndrome seen in dogs, and that the ammonia was derived from the blood in the alimentary tract. While blood ammonia determinations are required in the diagnosis and management of unusual cases, they are not required in the routine treatment of hepatic coma.

The Diagnosis of Circulating Anticoagulants

R. M. HARDISTY (St. Thomas's Hospital, London) said that circulating anticoagulants include inhibitors of blood thromboplastin formation and a much less common group of heparin-like substances. The latter might be distinguished by their neutralization by protamine sulphate or toluidine blue.

Inhibitors of thromboplastin formation are found in three groups of individuals: patients with haemophilia or Christmas disease, women during the first 12 months following a pregnancy, and a miscellaneous group of elderly people. In all of these cases, the anticoagulant appears to inhibit the reaction between antihaemophilic globulin and the Christmas factor.

The clinical features of the first of these three groups are indistinguishable from those of uncomplicated haemophilia. The last two groups are characterized by the spontaneous development of large haematomata, often accompanied by haemorrhage into the alimentary tract, in patients with no previous or family history of abnormal bleeding.

The whole blood clotting time is usually prolonged in these cases, and the one-stage prothrombin time and platelet count are normal. Prothrombin consumption is impaired.

The demonstration of an anticoagulant depends on the fact that the patient's blood interferes with the coagulation of normal blood to which it is added. This inhibitory effect may be demonstrated by the use of various techniques, involving tests on mixtures of normal plasmas or sera. These techniques include the determination of the clotting time, prothrombin consumption index, and thrombin generation of such mixtures, and the thromboplastin generation test. In this test, both alumina plasma and serum can be shown to contain the inhibitor.

The Diagnosis of Haemophilia and Christmas Disease

ROSEMARY BIGGS (Oxford) said that the diagnosis of both haemophilia and Christmas disease is based on both clinical and laboratory evidence. The laboratory tests of clotting function that have been used for the diagnosis of haemophilia and Christmas disease are the whole blood clotting time, the prothrombin consumption test, and the thromboplastin generation test. In haemophilia the clotting time is prolonged in more than half of the patients, but patients who are quite severely affected may have a normal clotting time. Similarly, the prothrombin consumption test usually gives abnormal

results with the blood of haemophilic patients, but with some samples normal results are obtained. The thromboplastin generation test gives more reliable results with haemophilic blood than either of the other tests, and using this technique it is possible to distinguish patients with haemophilia from those with Christmas disease. About 10% of patients previously diagnosed as having haemophilia are found to have Christmas disease. Of the patients with Christmas disease, many are rather mildly affected and have a normal clotting time and consumption of prothrombin.

Investigation of Haemorrhagic States with Special Reference to Defects of Coagulation of the Blood

E. K. BLACKBURN (Sheffield) described a method of investigation of patients with suspected haemorrhagic disorders. He said that consideration of the symptomatology was invaluable in answering the following four main questions: (1) Is a haemorrhagic state present? (2) If a haemorrhagic state is probably present, is there probably a coagulation defect or probably a capillary and/or a platelet defect? (3) If a coagulation defect is present, is it hereditary? (4) If a coagulation defect is present, is it acquired?

Laboratory tests used to exclude capillary and/or platelet defects were mentioned, and a simple theory of blood coagulation given.

The laboratory differentiation of coagulation defects is based in the main upon the one-stage prothrombin test and the thromboplastin generation test. If the former is normal one scheme of tests is followed, while if it is abnormal another scheme is used. In this way it is possible to differentiate between haemophilia, Christmas disease, cases with circulating anticoagulants, factor V, factor VII, and true prothrombin deficiencies, fibrinopenia, afibrinogenaemia, fibrinolysis, and thrombasthenia.

[This paper will shortly be published as a broadsheet. —ED.]

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BOOK REVIEWS

Clinical Bacteriology. By E. Joan Stokes. (Pp. vii+288. 20s.) London: Edward Arnold. 1955.

The work of the hospital bacteriologist has increased considerably in volume and complexity during recent years, due mainly to the widespread use of chemotherapy and the increasing recognition of the many opportunities for cross-infection in the hospital environment. A wide range of bacteria may be encountered and the methods of investigation often present a serious problem, particularly to the inexperienced worker. There is consequently much scope for the introduction of unsatisfactory techniques, which provide misleading information and so bring discredit on the clinical pathologist.

These dangers are obviously appreciated by Dr. Joan Stokes, who, in this book, reviews the field of clinical bacteriology and then describes methods which have proved satisfactory in routine hospital practice. General principles receive well-merited attention, and, while it is emphasized that each investigation should be treated as an individual problem, the importance of an early report is recognized. The value of microaerophilic and anaerobic techniques is repeatedly stressed and it is surprising that Brewer's medium is not specifically mentioned. The chapter on the identification of bacteria suffers from the attempt to condense this wide and complex subject into 48 pages; some clinical bacteriologists will be surprised, and probably distressed, to find that members of the *Salmonella*, *Shigella*, *Proteus*, and *Pseudomonas* groups are included in the term "coliforms," which is often used in a more restricted connotation. The technique of the serological tests is given in some detail, but the interpretation of the results, which often presents a serious problem to the bacteriologist, receives only brief mention. There is a short account of the pathogenic fungi, but the investigation of the virus diseases is not discussed. The laboratory aspects of chemotherapy are considered at some length, but the sulphonamide sensitivity tests could, with advantage, be treated separately as these are more difficult to perform and to interpret than the antibiotic tests. As might be expected, there is an excellent section on hospital cross-infection.

While some details of the different techniques may not receive general approval, this book contains much useful advice and information. It can be recommended for general guidance to all clinical pathologists and senior bacteriological technicians.

R. W. FAIRBROTHER.

An Introduction to Medical Laboratory Technology. By F. J. Baker, R. E. Silverton, and Eveline D. Luckcock. (Pp. x+330; one colour plate, 108 text figures. 30s.) London: Butterworth & Co. 1955.

The authors are members of the technical staff of the Brompton Hospital laboratory, and the text reflects their extensive experience. It provides the technical information required for the Intermediate Examination of the Institute of Medical Laboratory Technology. Apparatus is clearly described and illustrated, and the technical methods are well set out with all necessary detail. The contents include useful chapters on glass-blowing, microscopy, and the care of animals. Those concerned with teaching student-technicians will sympathize with the authors' attempt to remedy the defects of school science education by the inclusion of a chapter on the elementary laws of chemistry and the gas laws. The account of logarithms in this chapter seems in need of expansion if the uninitiated are to calculate successfully.

In an appendix on first aid, advice is given to the student unfortunate enough to swallow a culture, but little is said throughout the book about the less obvious ways in which a technician may infect himself or his colleagues. The precautions to be observed in dealing with potentially infected specimens should be impressed on the student from the beginning, and it is a pity that the authors do not give more attention to this point.

There is a very good glossary in which prefixes are sensibly included, and the production is excellent. This book deserves a good reception from students—and their teachers. Since it provides for their needs under one cover, it is not unreasonably priced.

A. J. MCCALL.

Studies on Fertility. Edited by R. G. Harrison. (Pp. ix+151; illustrated. 21s.) Oxford: Blackwell Scientific Publications. 1954.

This well-produced book contains a selection from the papers presented at the Sixth Annual Conference of the Society for the Study of Fertility, which exists to bring together all those whose work is concerned with some aspect of reproduction, whether medical, veterinary, or purely scientific. In this review it is only possible to indicate the subjects of the papers.

The veterinary side is represented only indirectly in the present volume. Professor R. G. Harrison, the editor, contributes an anatomical study of the effects on the vascularization of the testis when bulls of English breeds are subjected to South African climatic conditions. Bishop reviews the physiological reaction of bull spermatozoa to different types of diluent used in the storage of semen.

The scientific papers include three on the physiological, anatomical, and histological effects of various experimental treatments on the reproductive system of the rat (Clegg, Macdonald and Harrison, and MacMillan), two on the physiological action of spermicidal substances (Mann, Parkes, *et al.*), and one on the chemical composition of the fluid in the rabbit blastocyst and its relation to substances in the maternal blood stream (Lutwak-Mann).

The medical papers cover a wide range. G. I. M. Swyer describes a misleading testicular biopsy from a hypogonadal man, and J. K. Russell gives an analysis of medical histories in sub-fertile men, finding no cause for the condition in half of the 120 cases.

Dorothy M. Shotton discusses the outcome of pregnancy in 472 previously infertile women, and finds that the figures compare favourably with those for primigravida in general. Uterine tubal spasm appeared to be the only infertility factor correlated with an increased abortion rate and disordered uterine action during labour.

The value of the precise timing of ovulation by means of his rat ovary hyperaemia test is discussed by Dr. E. J. Farris, of the Wistar Institute; his paper suggests that, provided rats of the Wistar strain are used, the best time for coitus or for doing artificial insemination can be predicted with considerable accuracy.

Other papers are those by J. Lowenstein describing a coital training apparatus to help the impotent male; by Mary Pollock on the use of a 30% watery solution of sodium acetrizoate for hysterosalpingography; and by R. Palmer on the value of coelioscopy in the diagnosis of sterility.

In the last papers G. E. S. and H. W. Jones (Johns Hopkins Hospital) mention some interesting clinical syndromes associated with changes in the adrenals ranging from congenital pseudohermaphroditism to cases where hirsutism, oligomenorrhoea, and infertility became manifest after puberty, either with or without increased urinary excretion of 17-ketosteroids. They find quite small doses of cortisone (25 mg. daily) of value in dealing with these cases, regular menstruation being established and more than 50% of the previously infertile patients becoming pregnant.

CLARE HARVEY.
MARGARET H. JACKSON.

Serological Approaches to Studies of Protein Structure and Metabolism: Annual Conference on Protein Metabolism, 1954. Edited by William H. Cole. (Pp. 88. $2.00.) New Brunswick, New Jersey: Rutgers University Press. 1955.

This symposium comes at a most opportune moment. The advances it discusses are of equal interest to students of immunology, experimental biology, and protein chemistry. The papers dealing with the serological attack on protein orientation are scattered through the literature and liable to be overlooked by workers in one particular field.

The contributors to the symposium have presented the material they have to offer with clarity, and Dr. Boyden, as well as contributing to the programme, helped to gather together a group covering such a wide range and is to be congratulated on the part he played in producing this well-balanced volume.

Haurowitz and Heidelberger are such distinguished contributors in their fields that the articles on proteins as antibodies and antigens and on the effects of denaturation on the precipitin reaction, though they contain little new information, must command interest and respect. Gitlin's article, a veritable Cook's tour, shows how the precipitin reactions may be applied to clinical research into protein disposal. He touches on the use of "light scattering methods" to refine the precipitin reaction itself. Cohn devotes the major portion of his article to a study of the formation of galactosidase in *Bact. coli*. The wider implications of this data and the problem of enzyme synthesis are the most exciting parts of this book.

Munoz's contribution on the use and limitations of serum and agar techniques should be read by the host of workers who daily use this method.

This is not a book for beginners.

N. H. MARTIN.

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POST-TRAUMATIC ADRENAL APOPLEXY

BY

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The first description of haemorrhage into the adrenal glands is attributed to Griselius, of Vienna, in 1670 (Doran, 1907). In the nineteenth century cases, mostly of obscure aetiology, were described by Rayer (1837), Dickinson (1863), Mattei (1883), Carrington (1885), Churton (1886), and others, and Arnaud (1900) collected and reviewed 79 cases. Since then reports of patients with gross adrenal haemorrhage have been not infrequent, and the two clinico-pathological pictures of adrenal haemorrhage in the newborn and that associated with fulminating meningococcal septicaemia, now known as the Waterhouse-Friderichsen syndrome, have been separated. This has left many cases associated with diverse conditions. In some the haemorrhage has been spontaneous and unassociated with other disorders (e.g., Pearl and Brunn, 1928 ; Barsoum, 1936 ; Thorstad, 1942 ; Falconer, 1953). Occasionally extensive adrenal haemorrhage complicates severe burns (Dudgeon, 1904 ; Weiskotten, 1919 ; Harris, 1929 ; Snelling and Erb, 1935) ; sometimes it is associated with pregnancy or the puerperium (Hall and Hemken, 1936 ; Keele and Keele, 1942 ; Hurter, 1946 ; Dewhurst, 1951 ; Crawford, 1951) ; occasionally the condition complicates ulcerative colitis (Dr. P. N. Coleman, 1954, personal communication ; Wilson and Roth, 1953), agranulocytosis (Seligman, 1932), leukaemia (Lauckner and Hebbert, 1947), pneumonia, septicaemia, or cellulitis (Lusk and Brumbaugh, 1919 ; Seligman, 1932 ; Hall and Hemken, 1936). In other reports chronic visceral disease has been found (Simmonds, 1902), such as chronic pancreatitis (Lavenson, 1908), hydatid hepatic cyst (Godfrey, 1947), tuberculous pyelonephritis (Thorstad, 1942), and secondary carcinoma (Seligman, 1932), and occasionally fatal adrenal haemorrhage is said to complicate an abdominal operation (Taylor, 1930).

The first reports of cases following injury were those of Canton (1863) and Mattei (1883), but haemorrhage into the adrenals of guinea-pigs was observed by Brown-Séquard in 1852 after section of the spinal cord. In his review Arnaud concluded that only three out of 79 cases were due to trauma, and Lavenson (1908) could find only those reported by Canton and Mattei. In Pearl and Brunn's (1928) review there were no new cases attributed to injury, but since then at least seven cases have been reported, three by Seligman (1932) and one each by Chandler (1934), by Snelling and Erb (1935), by Thorstad (1942), and by Antonini (quoted by Thorstad).

The purpose of this paper is to describe 14 cases of unilateral or bilateral haemorrhage of the adrenal glands following severe injuries, to discuss the pathogenesis of this condition, and to attempt an assessment of its clinical and pathological significance.

The Patients

From 1948 to 1954, 50 necropsies were performed in the Birmingham Accident Hospital on patients who died after severe injuries to the chest or abdomen, and in 13 of these one or both adrenals were considerably haemorrhagic (26%). Another patient (Case 11) with severe lower limb injuries and only slight abdominal trauma also had a unilateral adrenal apoplexy, and these 14 patients form the basis of the present study.* The haemorrhage was bilateral in three subjects and unilateral in the other 11, nine of which involved the right adrenal.

Table I lists the age, sex, survival period, type of accident, and main injuries of each patient, together with any operation performed and significant complications. Thirteen patients were males, but this preponderance is probably occupational: 12 were adults, six under 40 years of age. Three fell from a height at work and the remainder were involved in road accidents with a motor or other vehicle. One patient died eight days after injury and the remainder within five days ; two of these died within 24 hours and five

*Among the 50 necropsies were two others in each of which a small central blood clot, 2-3 mm. diameter, was found in one adrenal. These glands have also been studied, as they show the minimal lesions which may occur.

TABLE I
SUMMARY OF TRAUMA IN 14 CASES

Case No.	Sex and Age (Yr.)	Survival Period	Mode of Injury	Main Injuries and Other Findings	Adrenal Apoplexy*
1	M 57	3 days	Fall from a height	Fractures 2 to 7 R. ribs; fracture-dislocation R. pelvis. Abdominal distension, vomiting. Uraemia (blood urea 280 mg.%)	Bilateral. R.—total. L.—partial (¼)
2	M 39	2 ,,	Run over by a lorry	Ruptured L. diaphragm. Fractures 7 to 11 L. ribs, haemothoraces. Fractures ilium, L. tibia, fibula, femur. Small laceration liver, torn jejunum, colon deperitonealized. (Op.—colostomy, etc.)	Bilateral R.—partial (¼) L.—partial (¼)
3	M 36	4 ,,	Pinned under a bus	Rupture L. kidney, spleen. Cracks R. and L. liver (op. splenectomy, liver packed). Low B.P. (80/40). Unconscious after 18 hours. Oliguria, uraemia (blood urea 210 mg.%). Cerebral ring haemorrhages ++	Bilateral R.—subtotal L.—partial (¼)
4	M 39	26 hours	Crushed between a lorry and a siding	Superficial tear L. liver (op. packed). Biliuria. Traumatic asphyxia. Myocardium bruised. Haemopericardium ++. Sudden collapse 3 hr. before death	R.—partial (¼)
5	M 21	1 day	Kicked by a horse, run over by cart	Gross rupture R. liver, torn hepatic vein. Haemoperitoneum +++. Rupture L. renal vein, bruised kidney. Fractures R. and L. ribs, bilateral haemothorax. Fracture humerus. (Op. liver packed)	R.—total
6	M 58	3½ days	Car accident	Fractures 9 to 11 L. ribs, L. haemothorax, rupture pleura. Multiple fractures calvarium, cerebral laceration. Medullary-cerebellar coning. (Op. removal comminuted fractures skull.) Sudden collapse day 2	R.—subtotal
7	M 78	4 ,,	Knocked down by a car	Fractures 7 R. rib, bloody pleural effusion. Multiple fractures pelvis, central dislocated R. hip. Medullary-cerebellar coning. (Op. cystotomy.) Collapsed twice day 3	R.—partial (¼)
8	M 70	3½ hours	Hit by a crane, fell from a height	Fractures R. 1 to 10 ribs, ilium, skull. Amputation both hands. (Op.—amputation completed.) Post-op. sudden death. Severe coronary atheroma	R.—partial (¼)
9	M 36	5 days	Fell off scaffold 25 feet. Hit chest and abdomen on way down	Ruptured R. liver ++. (Op.—laparotomy.) Fractures sternum, 2 to 9 R. ribs. Infected haemothoraces. Early peritonitis. Bronchopneumonia	R.—partial (¼) with diffuse haemorrhage
10	M 54	1¾ ,,	Knocked down by a car	Bilateral multiple fractures ribs, L. haemopneumothorax, small R. haemothorax. Superficial tear R. liver. Fractures skull and slight cerebral haemorrhage. Medullary cerebellar coning. Dislocated elbow. (Op.—reduced)	R.—total
11	M 38	2 ,,	Found lying in the road beside a truck	Bruising round duodenum, hepatic colon. Severe laceration and fracture L. thigh; perineal, anal laceration. Compound fracture R. tibia, fibula. Myohaemoglobinuria. (Op. L. thigh amputation, transverse colostomy.) Small myocardial rupture. (Myocarditis and fragmentation)	R.—total
12	F 55	21 hours	Knocked down by a car	Rupture spleen, L. kidney. (Op. nephrectomy.) Haemoperitoneum ++. Fractures 3 to 10 L. ribs, small haemothorax. Fracture humerus	L.—total
13	M 5	1½ days	Run over by a bus	Small L. haemothorax, torn pleura (ribs intact). Massive L. atelectasis. Capsule tear spleen. L. kidney bruised. Fracture pelvis. Severe laceration L. thigh, perineum. (Op. repair of laceration)	L.—partial (¼)
14	M 72	8 ,,	Knocked down by a cyclist	Multiple fractures R. ribs, cracks R. liver, ruptured right kidney. (Op.—liver suture.) Post-op. paralytic ileus (recovered). Bronchopneumonia. Infarct L. kidney	R.—partial (¼)

* In total and partial apoplexies the whole or part of the medulla respectively is occupied by haemorrhage (see text). The fractions indicate the medullary area involved.

on the second day. The mode and nature of many of the injuries indicated that the abdomen or thorax had been crushed.

Thoracic and Abdominal Injuries. — Closed abdominal or thoracic injuries were found in all 14 subjects, in 13 of whom the injuries were severe. Rib fractures on one or both sides were found in 10 and were usually associated with a unilateral or bilateral haemothorax; in two patients a pneumothorax was also present. One patient had a ruptured diaphragm, in another the heart was bruised and a haemopericardium later proved fatal (Case 4). In eight subjects the abdominal viscera were injured, seven of whom also suffered a chest injury: the liver was ruptured or cracked in seven (the main hepatic vein was torn in the one who received 52 pints of blood), the spleens of three were ruptured or torn, and in one patient the

bowel was also torn and partly deperitonealized. In five subjects one or other kidney was ruptured or bruised. In one patient (Case 11) abdominal injury was confined to bruising around the duodenum and hepatic colon.

Other Injuries.—These were often severe and multiple. Significant pelvic injuries were found in five patients who also received injuries to the chest or abdomen. The pelvis was badly fractured in three patients, in two of whom it was the major injury (Cases 1 and 7), but the pelvic viscera were intact.

Three subjects had fractured skulls and cerebral laceration, and haemorrhage was the major injury in Case 6.

Other injuries included fractures of the long bones (Cases 2, 5, 11), guillotine amputation of the hands (Case 8), and severe lacerations of the perineum, thigh, etc. (Cases 11 and 13). The main injuries of Case 11 were to the perineum and lower limbs.

The Adrenals

Before describing the adrenals of the present series, two anatomical types of adrenal haemorrhage must be distinguished. In the first a blood clot occupies part or the whole of the medulla and the cortex is stretched around it. The gland is enlarged and distorted in its transverse axes and may be 2 cm. or more thick (Fig. 1). This kind,

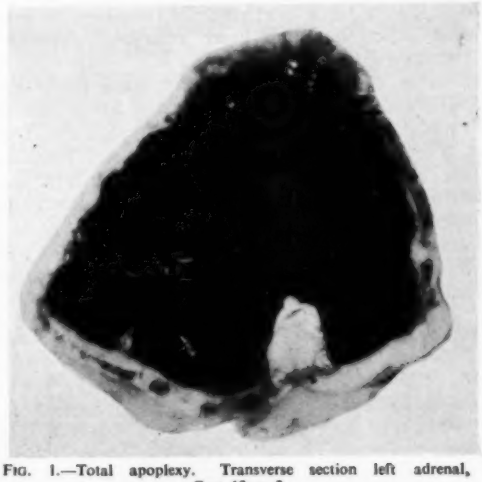

FIG. 1.—Total apoplexy. Transverse section left adrenal, Case 12, × 2.

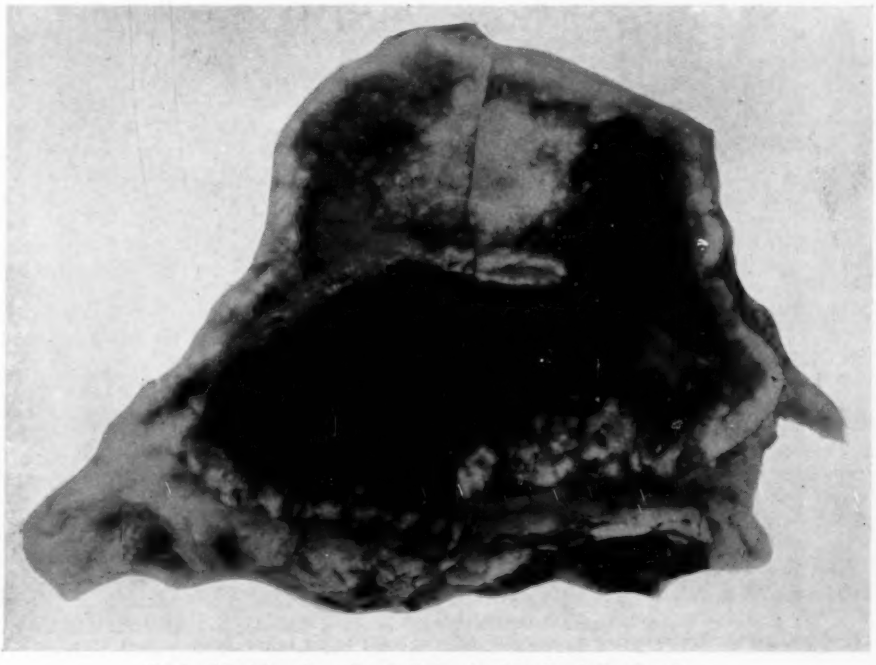

FIG. 2.—Subtotal apoplexy. Longitudinal section right adrenal, Case 3, × 2.

with which the present account is concerned, will be referred to as the centrally haemorrhagic or apoplectic gland. In the second type multiple focal haemorrhages, which may coalesce, occur in the medulla and cortex, but the gland retains its normal shape. This kind was not found in the present series but has been described elsewhere (Sevitt, 1955b).

In the present series in every gland the essential lesion was a central, firm, clotted haemorrhage, usually thickest in the centre, occupying a part or the whole of the medulla (Figs. 1 to 3). The clot, which distended the gland antero-posteriorly, was surrounded by the narrow, sometimes thinned and stretched cortex, which it frequently invaded and sometimes ruptured. In one patient the clot was thickest eccentrically (Case 8), but otherwise the thickest part of the haemorrhage was in the middle. Tiny focal haemorrhages were also visible in parts of the cortex. The central haemorrhage varied in size. Sometimes it occupied a quarter or less of the medulla. In these partial apoplexies the unaffected part of the gland retained its normal shape (Fig. 3). In other adrenals the central clot occupied the whole of the medulla: such apoplexies were total (Fig. 1). Transversely sectioned through the thickest part of the haemorrhage, the blood clots varied from 7 to 15 mm. in diameter in the partially apoplectic glands and from 20 to 25 mm. in those totally apoplectic.

In the bilaterally affected glands the right apoplexy was total in Case 1, subtotal in Case 3 (three-quarters of the medulla was filled with clot), and partial in Case 2, in which about half the medulla was occupied. Within the left glands the clots were smaller, involving no more than a

FIG. 3.—Partial apoplexy. Transverse sections of two parts of the right adrenal of Case 6. The central medullary haemorrhage has ruptured the cortex and has produced peri-adrenal haemorrhage, × 2.

quarter of the medulla. Four of the unilaterally affected adrenals were totally apoplectic (Cases 5, 10, 11, and 12), one subtotally (Case 6), and the remaining six were partially affected. In one of these (Case 9) the clot had ruptured through the cortex and a diffuse haemorrhage was present throughout most of the organ.

Periadrenal Haemorrhage and Rupture of the Cortex.—Haemorrhage or bruising of the periadrenal tissues of 10 glands was visible through the posterior peritoneum. This haemorrhage was never gross: it was always less than a centimetre thick and in four cases was merely a bruising of the periadrenal fat. In four patients it was associated with a local rupture of the cortex (Fig. 3) through which the central and external haemorrhages communicated (Cases 3 (right gland), 4, 6, and 8). Since similar haemorrhage was also found outside the intact glands and sometimes in other injured patients, it can arise independently of intra-adrenal haemorrhage.

Histology.—The right and left glands were fixed in 10% formol saline and paraffin sections were cut at 5μ. These were stained with haematoxylin and eosin, by Perl's method for iron, by Weigert's fibrin method, and Verhoef's and Van Gieson's techniques for elastica, smooth muscle, and connective tissue of blood vessels.

The central blood clots which had completely or almost completely destroyed the medullary tissue were formed of densely packed erythrocytes lying within an irregular and often poor fibrin network. The haemorrhage was recent: red cell dehaemoglobinization, haemosiderin pigment and organization of the periphery of the clot were absent. In some glands a dense, narrow layer of leucocytes near the edge of part of the clot was externally layered by further haemorrhage, suggesting that the bleeding may have occurred in two stages.

The central haemorrhage irregularly invaded the cortex, in some places as far as the capsule, in others to the zona reticularis or fasciculata. The boundary of the haemorrhagic area was often infiltrated by polymorphonuclear leucocytes which sometimes invaded the reticularis where the haemorrhage was confined to the medulla.

Discrete, often multiple foci of cortical haemorrhage were also seen.

Cortical necrosis was either associated with haemorrhage or was free of haemorrhage. Haemorrhagic necrosis was more extensive in some (e.g., Cases 6, 10, and 12) than in others (e.g., Cases 7, 8, 14). The necrotic areas were usually coextensive with the haemorrhage, sometimes extended beyond it, and some discrete foci were found histologically remote from the central clot.

Foci of necrosis without haemorrhage were also present in every gland. In some places the zona reticularis and inner fasciculata bordering the central clot were necrotic; often irregular focal zones of necrosis involved the fasciculata or less commonly the glomerulosa. The epithelial cells were necrotic, but the cortical architecture was often intact and the sinusoids sometimes congested. In the affected parts the epithelial cell nuclei had usually disappeared, but occasionally they were dense and pyknotic: the cytoplasm was either uniformly eosinophilic or had been partially disintegrated by multiple vacuoles. Many necrotic areas were loosely infiltrated by polymorphonuclear leucocytes which were denser at the periphery. In some an eosinophilic, albuminous exudate was present, particularly in zones bordering the central haemorrhage, and in one gland the exudate was partly fibrinous (Case 1, right gland). Some necrotic areas were bordered by haemorrhage, others by normal cortex. The necrosis was probably the result of ischaemia from local pressure or near-by haemorrhage. However, in every gland much of the cortex was normal, in some the major part.

Pathogenesis of Post-traumatic Adrenal Apoplexy

Most of the bleeding comes not from the solid cortex but from the looser medulla and juxtamedullary cortex. It will be shown that rupture of small vessels, mainly sinusoids and venules, is probably brought about by a temporary compression of the gland.

The Vessels.—The adrenal has a rich blood supply from three or more arteries which pass through the capsule and break up into a sinusoidal network in the cortex. Drainage is effected through a single emissary vein which passes through the medulla, where it is joined by tributary vessels which drain the medulla and the juxtamedullary cortex.

Medium-sized and small venules were always found at the periphery of the clot, usually in the juxta-medullary cortex, and occasionally within the clot. In 12 of the 17 glands small tears, fissures, or ruptures were present in the walls of venules possessing a smooth muscle coat and in endothelial-lined channels. Similar ruptures were seen in the two glands containing the small central clots referred to in the footnote on page 185. Either part or the whole thickness of the wall was

torn and a trickle of red cells could often be seen in the gap. The venous walls were often oedematous, occasionally necrotic, and sometimes infiltrated by polymorphonuclear leucocytes.

Venous thrombosis has been stressed by various writers as the important factor producing adrenal haemorrhage (e.g., Hall and Hemken, 1936 ; Keele and Keele, 1942). In the present series venous thrombi were found in only one gland (right gland, Case 2). Here a few medium-sized veins contained hyaline thrombi, but torn venules were also present. The venous thrombosis probably developed after the haemorrhage had occurred. In two glands hyaline thrombosis of some cortical sinusoids was present which must also have been post-haemorrhagic. The arteries and arterioles were intact and never thrombosed.

Mechanism of the Apoplexy.—Deeply hidden behind the abdominal cavity and protected by the liver and other organs in front and by the spinal column behind, the small adrenals are considerably protected from the effects of violence. For externally applied forces to act on an adrenal they must pass through and act upon the surrounding structures and organs. These tissues are likely to be injured if the adrenal becomes haemorrhagic. Therefore if the apoplexy is due to an anteroposterior crushing injury the haemorrhagic adrenal and injured viscera would be found on the same side. An alternative possibility is that haemorrhage is the result of an acute rise of intra-adrenal venous pressure resulting from sudden and severe compression of the inferior vena cava during the crushing injury.

Consider Case 12, a woman in whom a left adrenal apoplexy was found. She was knocked down and probably run over by a car ; the spleen and left kidney were ruptured and the left thoracic cage fractured. Both the adrenal haemorrhage and the main thoraco-abdominal injuries were on the left side in this patient and in Case 13. Of the nine patients with right-sided apoplexy, eight had thoraco-abdominal injuries and in six major ipsilateral injuries were present (Cases 5, 7, 8, 9, 10, and 14). For example, in Case 14, the right ribs were fractured, the right side of the liver was cracked, the right kidney was torn, and the right adrenal was haemorrhagic. In Case 4 the bruising of the heart was probably due to crushing of the chest.

In one patient (Case 6) there was an apparent contradiction between the left-sided chest injury and the right-sided adrenal apoplexy. His apoplexy may have been the result of oblique forces transmitted to the right side of the liver, which might then have crushed the adrenal against the vertebral column. Oblique forces might also explain the apoplexy of Case 11, in which the evidence of thoraco-abdominal trauma was confined to bruising around the duodenum and hepatic colon.

Of those with both glands haemorrhagic, Case 1 fell from a height and had severe right-sided injuries, Case 2 was run over by a lorry and extensive crush injuries of the chest and abdomen were present, and in Case 3, a man who was pinned under a bus, the upper abdomen was crushed and injured.

Therefore the trauma in most patients corresponded with the side of the adrenal apoplexy and crushing injuries could be implicated. The conclusions cannot be escaped that post-traumatic adrenal haemorrhage is the result of direct violence.

Blood Transfusion.—Incompatible transfusion has been incriminated in some cases of adrenal haemorrhage (Snelling and Erb, 1935 ; Hurter, 1946 ; Crawford, 1951). On admission the patients were shocked and were urgently transfused. In four subjects one to four bottles of Group O Rhesus-negative (D-negative) blood were given first and were followed by blood of the homologous ABO and Rhesus (D) type. The remainder received only homologous blood after rapid ABO grouping and Rhesus typing, the results of which were confirmed by a slower method. Cross-matching of the first bottles of blood with the patient's serum was not possible before transfusion but was always performed later. All the cross-matches were carried out in both saline and albumin suspension at 37° C. and no incompatibility was detected.

Four of the patients received two, three, or four bottles of blood ; six were given 10 to 12 bottles and the remainder 6, 16, 21, and 52 bottles respectively. There was no clinical or laboratory evidence of adverse reaction. The haemoglobinuria found in Case 11 was due to myohaemoglobin.

Significance of Adrenal Apoplexy after Trauma

All the patients were severely injured and only in Cases 4 and 8 was death sudden and unexpected. Necropsies revealed a haemopericardium in the former and severe coronary atheroma in the latter. The post-mortem findings in Cases 5, 6, and 9 indicated that the injuries were too severe to expect survival, and that the death of Case 12 was due to haemorrhage from a ruptured spleen. Both Cases 7 and 14 were over 70 years of age. In Cases 10, 11, and 13 adrenal haemorrhage was judged to

have a possible but doubtful clinical significance, and in the remaining three it may have contributed to the deaths of the patients, since the lesions were bilateral (Cases 1, 2, 3). However, the fatal outcome of even these patients was not surprising, since not only were the injuries severe but two of them developed renal failure (Cases 1 and 3) and the other had had intestinal injuries (Case 2). Nevertheless the adrenal haemorrhage may have produced its own physiopathological disorder and have been responsible for the abdominal distension and vomiting in Case 1, the low blood pressure in Case 3, and the sudden collapse of Cases 6 and 7. The possibility that the adrenal haemorrhage played a contributory role in the deaths of these and other patients cannot be excluded on clinical grounds.

Histological Tests of Adrenocortical Hyperactivity

The influence of adrenal haemorrhage on cortical activity might be assessed by two histological tests. One of these is based on the number of eosinophil leucocytes in the spleen (Sevitt, 1955a, 1955b) and the other upon the depletion of cortical lipoid in the hyperactive adrenal cortex.

Splenic Eosinopenia.—Elsewhere it has been shown that the normal spleen contains many eosinophil leucocytes, that within 12 to 24 hours of injury eosinopenia occurs and is found in those who die during the next few days (Sevitt, 1955a; Sevitt, 1955b). It was also found that splenic eosinopenia reflects blood eosinopenia, and, since the latter is due to adrenocortical hyperactivity, the former was postulated as also reflecting this activity. Splenic eosinopenia therefore may be used as post-mortem evidence of adrenocortical hyperactivity. The normal range of eosinophils in the spleen varies from 200 to 700 per 100 high power (H.P.) fields (×378) in 5μ paraffin sections and never falls below 100 per 100 H.P. fields. Values below 100 indicate eosinopenia. In Fig. 4 the shaded area represents the limits of the expected values after severe trauma. Up to a few hours of injury the normal range of splenic eosinophils is always present: thereafter and up to 24 hours after trauma—the transition period—either normal, intermediate, or definite eosinopenic values may occur.

Paraffin blocks of spleens were available in nine of the 14 cases. Sections were cut at 5μ and were stained by carbol-chromotrope and haematoxylin (Lendrum, 1944) which coloured the eosinophil granules a bright red. The number of eosinophil leucocytes in 200 H.P. fields from two sections was counted and the results expressed per 100 H.P. fields.

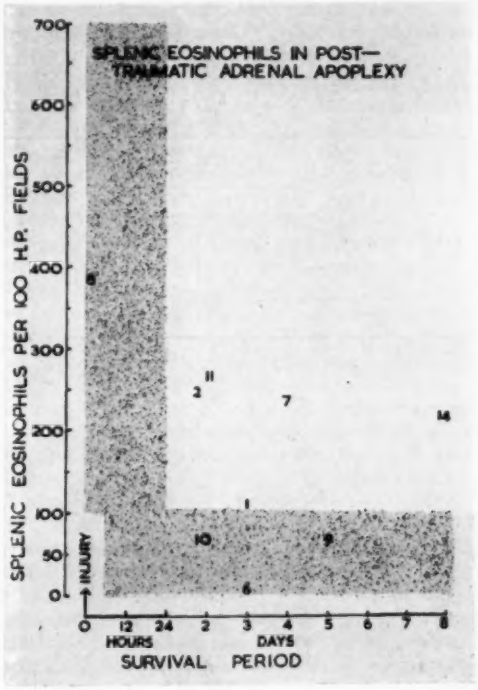

FIG. 4.

Depletion of Cortical Lipoid.—The normal resting adrenal cortex contains abundant sudanophil lipoid in all its zones. Depletion of lipoid from the human adrenal occurs after severe haemorrhage and injury (Elliott, 1914; Urechia, Manta, and Bumbacescu, 1940), and is usually associated with splenic eosinopenia (Sevitt, 1955b). Significant loss of cortical lipoid will therefore be interpreted as indicating adrenocortical hypersecretion. Conversely, if lipoid depletion, which is expected after injury, does not occur, its absence will be interpreted as indicating absence of adrenocortical hypersecretion.

In 12 of the 14 cases both adrenal glands were available for study. Frozen sections were cut at 10 to 15μ, stained with Sudan IV or oil red and counterstained with haematoxylin or light green. Adrenocortical depletion of lipoid was assessed histologically as 0, trace, +, ++, and +++ (see footnote of Table II).

Results.—These are shown in Fig. 4 and Table II.

In Cases 6, 9, and 10 splenic eosinopenia was present with values of 3, 74, and 77 eosinophils per 100 H.P. fields. These figures indicate adrenocortical hyperactivity. This was supported by the finding of considerable (++) depletion of cortical lipoid in the intact, non-haemorrhagic glands of these three patients (also in the apoplectic glands of Cases 6 and 9).

TABLE II

SPLENIC EOSINOPHIL CONTENT AND LOSS OF ADRENOCORTICAL LIPOID IN 10 SUBJECTS SURVIVING MORE THAN 24 HOURS

Spleen	Degree of Adrenocortical Lipoid Depletion		
	0 or Trace	++	+++
Eosinopenic (0–100 per 100 H.P. fields) ..		6, 9, 10	
Doubtful ..		1	
Eosinophilic (>200 per 100 H.P. fields) ..	7, 14	2	11
Not available ..		3, 4	

Numerals refer to case numbers.
++ depletion indicates 25% to 75% of cortex affected.
+++ >75%.

Splenic eosinopenia was not found in Cases 2, 7, 11, or 14. The splenic eosinophils were 242, 235, 262, and 210 per 100 H.P. fields respectively, all of which are within the normal range. The significance of post-traumatic splenic eosinophilia is less certain than that of splenic eosinopenia, since the former is a normal phenomenon. Splenic eosinophilia also occurs in Addison's disease (Sevitt, 1955b), but nevertheless it cannot be used in any particular case as evidence of adrenocortical failure. However, it may be used in a special group of cases such as the present one. The finding of eosinophilic values in the spleens of four out of eight patients (excluding Case 8) may be significant, since they died at a time when splenic eosinopenia would have been expected. In these patients adrenocortical activity may have been absent. In two patients, Cases 7 and 14, there was little or no depletion of cortical lipoid in either the haemorrhagic or intact glands, suggesting that there had not been any cortical hypersecretion. In these patients the combined evidence of splenic eosinophilia with lack of lipoid depletion indicated that adrenocortical failure may have occurred. This finding is unexpected not only because the adrenal apoplexies were unilateral but also because the central blood clots involved relatively small areas of the medulla (Table I). Inhibition of cortical hyperactivity, reflex or otherwise, of both intact and haemorrhagic glands must have occurred, since the absence of hyperactivity of both glands cannot be due to the direct effect of haemorrhage.

In Case 11, however, there was great loss (+++) of cortical lipoid from the intact adrenal (although the loss from the haemorrhagic gland was considerably less (trace or +). A considerable lipoid loss was also found in the lesser haemorrhagic gland of Case 2. In both these patients there is an apparent paradox between the splenic eosinophilia and considerable adrenocortical loss of lipoid. In the absence of other evidence the latter would appear to be the most direct evidence of hyperactivity and therefore these glands may be considered to have been in a hyperactive state at the time of death.

In Cases 1 and 3, which were bilaterally apoplectic, cortical lipoid loss was also considerable (++) in the lesser of the two haemorrhagic adrenals and indicated hyperactivity of the glands. In the former a borderline value of 104 splenic eosinophils per 100 H.P. fields was found. The considerable depletion of lipoid in the intact gland in Case 4 may also be considered as evidence of adrenocortical hyperactivity (spleen section not available).

In Case 8 a normal value of 388 eosinophils per 100 H.P. fields was found in the spleen and cortical lipoid depletion of both glands was slight. No conclusion can be reached as to the significance of these findings, since the patient died three and a half hours after injury, which was not sufficient time either for splenic eosinopenia or lipoid depletion to develop. Case 12 also died within 24 hours of injury and showed only slight evidence of cortico-lipoid depletion (spleen section not available). Neither of these cases is included in Table II.

Therefore evidence of adrenocortical hypersecretion based on depletion of cortical lipoid was found in eight out of 12 patients, in three or four of whom there was also splenic eosinopenic evidence of hyperactivity. In two patients both the adrenal lipoid and splenic eosinophil tests indicated absence of activity of both the normal and apoplectic glands and suggested that adrenocortical failure due to inhibition of the glands had occurred.

Discussion

Although few cases have been previously described, central haemorrhage into one, or less commonly both adrenals, is not an uncommon finding in patients who die after severe injury to the chest or abdomen, as it was found in 13 out of 50 necropsied patients. It was also present in

another severely injured patient with relatively minor abdominal trauma. It is likely that adrenal apoplexy also occurs in some of those who survive similar injuries, but at present those who are affected are not recognized and neither the incidence nor the clinical significance of the haemorrhage is known. Their recognition may be important, since any morbidity or fatality due to adrenal haemorrhage might be lessened by the administration of cortisone or other drugs.

The mechanism of the haemorrhage and its central location within the gland are probably dependent on the anatomical relationships and histological structure of the organ and the peculiarities of its blood supply. One theory, which is consistent with the data, depends on the following considerations. Parts of both glands lie in front of the spinal column against which they could be compressed when the upper abdomen or lower thorax is severely crushed. The right adrenal is more likely to be damaged than the left because much of its anterior surface is closely opposed to the liver. This could largely explain why nine of the 11 unilateral apoplexies involved the right gland. Damage to vessels occurs more readily within the spongy medulla and loosely textured zona reticularis than within the solid cortex. Rupture of the centrally situated venules and sinusoids occurs and a central haemorrhage develops which distends the peripheral cortex. Thus the typical result of injury to the adrenal is a central haemorrhage just as the classical result of trauma to the spleen or liver is a rupture. On the other hand, the central haemorrhage might follow an acute rise of intra-adrenal venous pressure resulting from sudden and severe compression of the inferior vena cava during the compression of the abdomen. The right adrenal would also be affected more often than the left because the right adrenal vein drains directly into the vena cava whilst the left vein first joins the renal vein.

Among Snelling and Erb's cases of adrenal haemorrhage, one followed a head injury, and cerebral injury was also found in two of Seligman's cases and in Cases 6, 8, and 10. The question arises whether cerebral injury, nervous stimulation, or shock can produce adrenal haemorrhage, as this might also explain the contralateral adrenal haemorrhages in Case 6 and in Seligman's Case 1, and also the apoplexy in Case 11, in which severe thoraco-abdominal injury was absent. On the other hand, the writer has never seen adrenal apoplexy in a not inconsiderable necropsy experience of cerebral, spinal cord, and other severe injuries, so that adrenal haemorrhage from nervous irritation or shock must be a rare condition if it occurs at all. It is therefore suggested that these apparent paradoxes are due to unsuspected mechanical stresses acting on the adrenal which reach it through a complex thoracic or abdominal pathway.

Any clinico-pathological significance attached to adrenal apoplexy is probably due to interference with cortical rather than medullary function, since only the cortex is essential to life. Cortical activity may be affected in two ways. First, parts of it are destroyed by haemorrhage or otherwise rendered necrotic, but since much remains histologically viable death of tissue probably plays a minor part in reducing cortical activity. Secondly, the pressure of the central haemorrhage could interfere with the venous drainage of the gland and abolish or reduce the venous blood flow, particularly when the clot fills the whole medullary space. Formation of hormones may cease, or, if the capillary blood flow persists, may continue for a time and depletion of cortical lipoid may occur even though the secretions never reach the inferior vena cava. This may have occurred, for example, in the totally apoplectic gland of Case 1 in which considerable depletion of cortical lipoid was found. In partially apoplectic glands the secretion from the hyperactive cortex will reach the general circulation, since drainage of the venous tributaries in the unaffected part of the medulla will continue unless the main vein is compressed near its place of exit.

Clinical assessment of the significance of the apoplexies was rendered difficult because all the patients were severely injured, and death was usually not surprising. However, the possibility that certain symptoms were due to the haemorrhagic adrenals and that acute adrenocortical failure played a contributory role in the death of some patients could not be excluded.

Two histological tests for adrenocortical hyperactivity, one based on eosinopenia of the spleen and the other upon the depletion of lipoid in the cortex, were of some value. Evidence of adrenocortical hyperactivity based on loss of cortical lipoid was found in eight out of 12 patients, in three (or four) of whom there was also splenic eosinopenia. Certainly in these three or four patients physiological failure of cortical activity had not occurred. On the other hand, the finding of splenic eosinophilia in four out of eight patients who died at a time after injury when eosinopenia would have been expected suggested that adrenocortical hyperactivity may have been

absent and that physiological failure may have occurred. In two cases the absence of scanty loss of cortical lipoid from both the intact and apoplectic glands supported this. As only one gland was haemorrhagic, activity of the cortex of the intact gland must have been inhibited. In the other two patients the considerable loss of cortical lipoid was local evidence of cortical hypersecretion, but in the bilaterally haemorrhagic subject the endocrine secreted may never have reached the systemic circulation because of possible pressure on the central adrenal veins. This cannot explain the association of splenic eosinophilia with severe loss of lipoid in the patient with unilateral adrenal haemorrhage, but a similar association occasionally occurs in patients with intact adrenals (Sevitt, 1955b).

Summary

Central apoplexy of one or both adrenal glands was found in 13 out of 50 patients who died after severe closed injuries to chest or abdomen (26%) and in another severely injured patient showing slight evidence of abdominal injury. Evidence is advanced that crushing of the glands tears the medullary venules and is responsible for the central haemorrhage. This stretches the cortex, often invades it, and sometimes ruptures it.

The significance of the apoplexies was estimated by two histological tests of adrenocortical hyperactivity, one based on the eosinophil content of the spleen and the other on depletion of adrenocortical lipoid, particularly in the non-haemorrhagic glands. Both were in agreement that cortical hyperactivity had been present in at least three or four patients. Evidence of hyperactivity based on cortical lipoid loss alone was found in eight out of 12 cases examined. The results of both tests suggested that adrenocortical failure due to glandular inhibition had occurred in two patients with unilateral apoplexy.

Addendum

During the first few months of 1955, necropsies were performed on 10 patients with closed thoracic, abdominal, or pelvic injuries and adrenal apoplexy was found in three of them. This makes a total of 16 out of 60 cases (approximately 27%). The first subject (male, 23 years), who died two days after a road accident, sustained multiple right rib fractures and a lacerated lung. He had an incomplete right adrenal apoplexy. Extreme eosinopenia of the spleen and partial depletion of lipoid from the left adrenal cortex indicated adrenocortical hyperactivity. In the second patient (male, 7 years) bilateral adrenal apoplexy appeared to have precipitated or accelerated his death. He was knocked down by a van, sustained a compound fracture of the right tibia and fibula and a ruptured spleen which was removed. He was transfused with 2 pints of blood. Next day, sudden collapse was associated with sudden tachycardia (pulse 160), respirations 40 and a fall of blood pressure to 90/60. Soon he became unconscious and died four hours after his collapse and about two days after injury. Necropsy revealed fractures of right ribs 9–12, a central haemorrhagic laceration of the right side of the liver with superficial tears of the left side, a slight bruise of the right frontal cerebral lobe, and a bilateral total adrenal apoplexy. Histologically there was complete haemorrhagic necrosis of the medullae and cortices and the latter were stuffed with lipoid. Unfortunately the spleen had been discarded. The third patient (male, 78 years) fell down the stairs and fractured the ninth and tenth right ribs. He died 10 days later ; severe coronary sclerosis, duodenal ulceration, an old pulmonary empyema, and a partial apoplexy of both adrenals (right more than the left) were present.

The writer is a part-time member of the scientific staff of the Medical Research Council.

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THE JAWS AND TEETH IN PAGET'S DISEASE OF BONE

BY

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That Paget's disease of bone may affect the jaws has been known since Moore's (1923) report of a case in which there was a bony tumour of the maxilla together with osteoporosis circumscripta of the frontal region, though until quite recently the disease in this site has been considered rare. Osteoporosis circumscripta itself has now been identified with an early phase of Paget's disease (Sosman, 1927; and others), and a number of cases have since been described in which there has been a coexisting lesion in the maxilla (Kasabach and Gutman, 1937; Elkeles, 1947; Rushton, 1948). It has also been realized that the symptoms arising from the lesions in the jaws may be the first of which the patient complains, leading to detection of lesions in other parts of the skull or in other bones (Novak and Burket, 1944; Jacobs, 1945), or that such lesions may be the only ones present (Thoma, Howe, and Wenig, 1945).

The incidence of jaw lesions is uncertain, as most of the published reports concern single cases or relatively small series, with the exception of the report by Stafne and Austin (1938), who found 23 cases with mandibular or maxillary involvement out of 138 cases in which the skull was affected. The maxilla is much more often involved than the mandible; in the series just referred to the mandible was involved in only three of the 23 cases. Instances of mandibular involvement have also been reported by Seldin (1933), Glickman (1943), Jacobs (1945), Thoma *et al.* (1945), and Stafne (1946).

The teeth are often affected in association with the surrounding bone, the principal abnormality being hypercementosis (Fox, 1933). As a result of enlargement of the jaws and of the overgrowth of cementum which may lead to ankylosis of the involved teeth and difficulty in their extraction, it not infrequently happens that the patient with Paget's disease is first seen by the dental surgeon. Thus the specimens which the pathologist receives for histological examination may be teeth or fragments of bone from the jaws, and it is the purpose of this paper to describe the appearances in these tissues when affected by Paget's disease. The materials from which these observations have been made comprise teeth and maxillary bone from six patients who complained of dental symptoms in the first instance.

Clinical Features

The patient with Paget's disease affecting the jaw complains of progressive enlargement of the maxilla or, much less frequently, the mandible. As a result of the increase in size in the dental arch the teeth become spaced, and if dentures are worn these cease to fit properly. The disproportion in size between the maxilla and the mandible gives rise to the inverted triangle type of facial contour.

The overgrowth of cementum on the roots of the teeth may lead to difficulty in extraction; the patient may be first referred to hospital on this account. Or following extractions persistent sinuses may develop, as the dense, avascular areas of bone which form in the osteosclerotic phase of the disease appear to be particularly prone to infection (Burket, 1946).

Pain occasionally occurs in connexion with the jaw lesions, sometimes of considerable severity.

Histological Observations

Bone.—Microscopic examination of bone from the jaws shows the characteristic changes of Paget's disease as seen in other parts of the skeleton. These are due essentially to a combination of destruction and repair, occurring without relation to the statics or dynamics of the involved bone (Weinmann and Sicher, 1947).

Teeth.—That the teeth may be involved in Paget's disease has been recognized since Fox's

FIG. 1.—The apices of two normal teeth *in situ* in the alveolus, × 9.

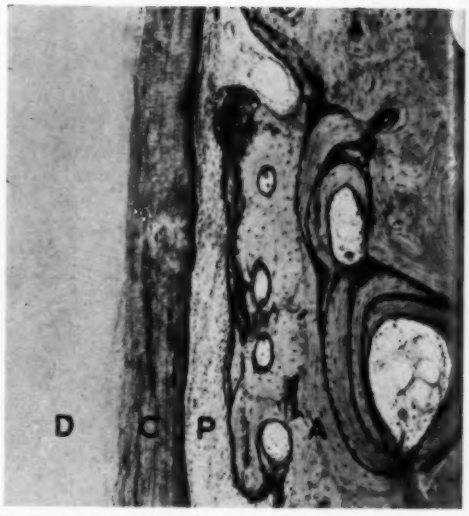

FIG. 2.—Higher magnification of a field from Fig. 1. D, dentine; C, cementum; P, periodontal membrane; A, alveolar bone, × 50.

FIG. 3.—Tooth showing hypercementosis due to infection, × 4.

FIG. 4.—Apex of tooth shown in Fig. 3, to demonstrate the lamellar arrangement of the cementum, × 50.

(1933) description of a case in which the maxilla was affected, and the roots of all the maxillary teeth showed, radiographically, the presence of knob-like irregularities. Stafne and Austin (1938) showed that hypercementosis of the teeth is a characteristic finding in Paget's disease, and many other authors have since reported similar findings. Descriptions of the histopathology of the teeth are scanty. Rushton (1938) reports the histological findings in three teeth from two cases of Paget's disease affecting the jaws, and Thoma (1950) also describes the teeth in two cases.

The Normal Tooth.—Fig. 1 shows the apical portions of two teeth, *in situ* in the alveolus, and demonstrates the nature of their attachment to the jaw. Fig. 2 is a higher magnification to show detail. The dentine, D, is lined by a thin shell of cementum, C, in which are embedded the connective tissue fibres of the periodontal membrane, P. This membrane acts as a suspensory ligament for the tooth, attaching it to the alveolus, A.

Cementum is a modified type of bone covering the roots of the teeth, two forms being recognized, acellular and cellular. Acellular cementum consists of calcified tissue in which is embedded the Sharpey's fibres of the periodontal membrane; it contains no cell spaces or cells. Cellular cementum contains cementocytes lying in lacunae, and is therefore similar to bone. Acellular cementum is normally laid down on the surface of dentine, as in Figs. 1 and 2, while cellular cementum is usually formed on the surface of acellular cementum, but the layers of the two types may alternate in almost any arrangement (Orban, 1949). Both types of cementum are separated by incremental lines.

Hypercementosis.—The term hypercementosis is used to denote an abnormal thickening of the cementum, which may be diffuse or may be confined to one part of the tooth only. Paget's disease is a relatively uncommon cause of the condition, which occurs frequently as a result of other factors, such as infection, but the presence of hypercementosis in a patient in the appropriate age group should always lead to consideration of the possibility of Paget's disease.

Fig. 3 shows an example of hypercementosis due to periapical infection. The apical two-thirds of the tooth is ensheathed in a relatively thick mass of cementum, laid down in lamellae. The incremental lines are clearly seen in the higher magnification of Fig. 4, and it will be noticed that these form a series of more or less regular layers. Nowhere is there any suggestion of mosaic pattern.

Another example of hypercementosis due to infection is shown in Figs. 5 and 6. Though the cementum has been deposited more regularly in this case than in the preceding specimen there is no evidence of the typical curvilinear markings of Paget's disease.

Paget's Disease.—The upper premolar tooth shown in Fig. 11 was obtained from a man aged 63 years, who complained of pain in the upper jaw following dental extractions seven weeks previously. He also complained of increasing deafness and tinnitus. On examination there was marked enlargement of the maxillary tuberosities and pronounced prominence of the labial maxilla. Radiological examination showed diffuse woolly sclerosis over much of the maxilla and the calvarium was thickened. The serum alkaline phosphatase was 59 units.

Histological examination of the tooth shows hypercementosis (Fig. 7). The increase in cementum involves both roots of the tooth and is diffusely distributed except at one point, where the hyperplastic tissue forms a spur-like projection. Immediately above this projection there remain some fibres of the periodontal membrane, containing two small fragments of alveolar bone. Higher power magnification (Fig. 8) shows the spur-like structure to consist of laminae of cellular and acellular cementum. These are laid down for the most part in quite regular layers, but at about the centre of the photograph there can be seen an area where the cement lines are irregular and pronounced, suggesting a mosaic appearance. One of the fragments of alveolar bone, seen in higher magnification in Fig. 9, likewise shows the curvilinear markings associated with Paget's disease.

These findings are of course minimal, and would of themselves hardly constitute sufficient evidence for a diagnosis of Paget's disease. Nevertheless, they are sufficient to arouse suspicion, so that full radiological and appropriate biochemical examinations are undertaken. A further biopsy was performed in this case, a portion of alveolar bone being removed. This showed the typical changes of Paget's disease.

The maxillary molar teeth shown in Figs. 10 and 11 were removed from a woman aged 73 years, who complained of pain in both upper canine regions, and of increasing deafness of some years' duration. Radiological examination showed gross hypercementosis of the maxillary molar teeth and the typical appearances of Paget's disease in the bones of the cranium. The serum alkaline phosphatase was 27 units.

Histological examination of the teeth reveals a striking appearance due to the masses of cementum which have been laid down around the roots. This tissue has been deposited in an irregular manner, quite unlike the lamellation which normally takes place. Figs. 12 and 13 are higher magnifications of the root area and cementum of the specimen seen in Fig. 11 and show the broad, deeply staining cement lines indicating the complicated cycle of deposition and resorption of cementum which has taken place. This activity has produced a picture quite different from that seen in the examples of cementum overgrowth due to infection shown in Figs. 4 and 6. In the former, the excessive cementum has been laid down in lamellae, while in the latter, though the lamellar

FIG. 5

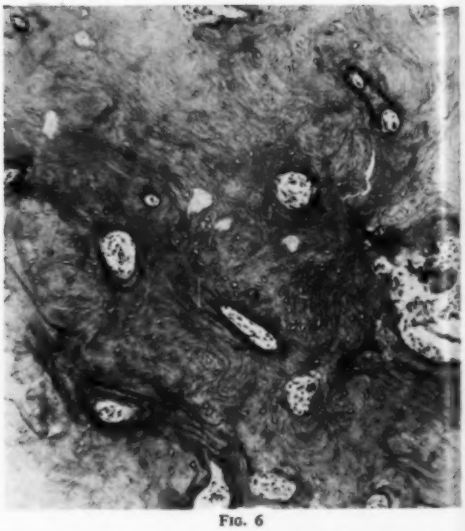

FIG. 6

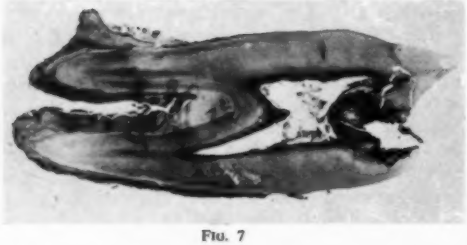

FIG. 7

FIG. 8

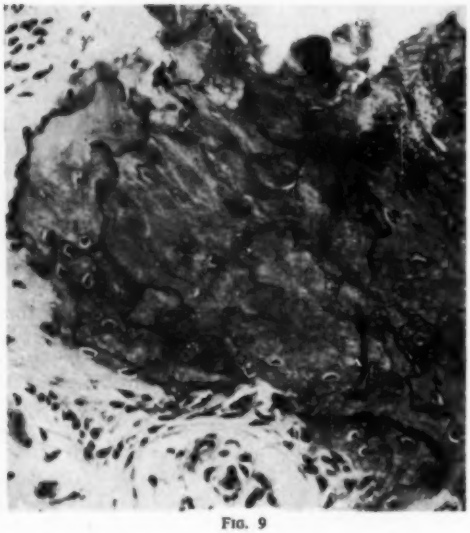

FIG. 9

FIG. 5.—Apex of tooth showing hypercementosis due to infection, × 9.

FIG. 6.—Higher magnification of a field from Fig. 5, × 50.

FIG. 7.—Tooth from a case of Paget's disease showing hypercementosis of both roots, with a spur-like projection in one area, × 3.5.

FIG. 8.—Higher magnification of the spur shown in Fig. 7. Irregular cement lines are seen, × 50.

FIG. 9.—Fragment of bone from the periodontal membrane of the tooth shown in Fig. 7. The cement lines show a mosaic appearance, × 200.

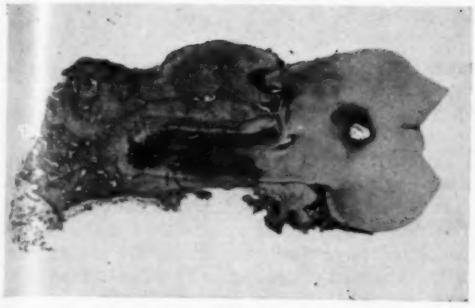

FIG. 10

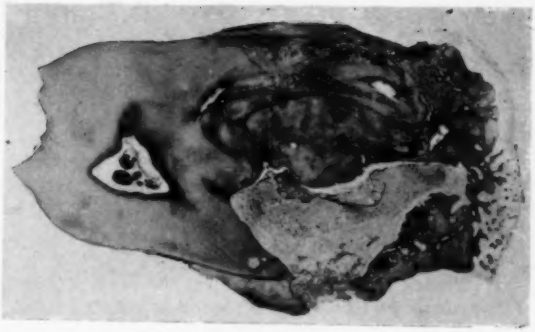

FIG. 11

FIG. 12

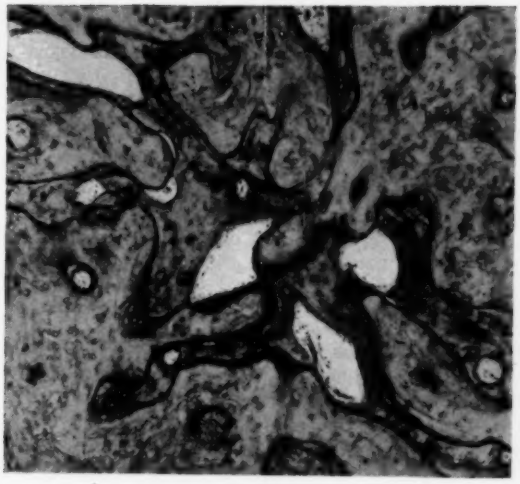

FIG. 13

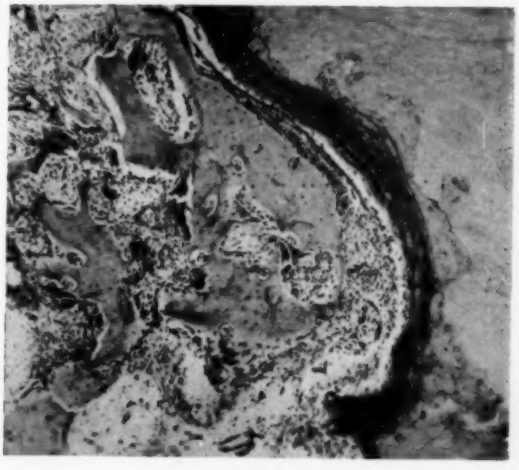

FIG 14

FIG. 10.—Marked hypercementosis in Paget's disease, × 3.

FIG. 11.—Marked hypercementosis in Paget's disease, × 3.

FIG. 12.—Field from Fig. 11 showing the irregular nature of cementum deposition, × 50.

FIG. 13.—Field from Fig. 11 showing broad cement lines and mosaic pattern in the cementum, × 50.

FIG. 14.—Field from Fig. 11 showing resorption of cementum and adjacent bone with marked osteoblastic and osteoclastic activity × 50.

arrangement is lacking, the thick mass of cementum does not show curvilinear markings.

Another feature in the pathological process is seen in Fig. 14. This is a field from Fig. 11, showing resorption of dentine with repair by comparatively normal cementum and ingrowth of active Paget bone.

Discussion

When Paget's disease involves the jaws, it may do so as part of a more general affection of the skull and other parts of the skeleton. In cases of this type the diagnosis is generally made on clinical grounds, together with the results of radiological and biochemical examinations. The pathologist may receive portions of bone for confirmatory examination, or sometimes bone is removed from the enlarged maxilla or mandible for prosthetic reasons.

In other cases the jaws alone may be affected, and the clinical and radiographic appearances may be interpreted as those of chronic osteomyelitis. In such cases histological examination of bone or teeth leads to the correct diagnosis or where the appearances are suggestive, if not conclusive, to the performance of biochemical tests and adequate follow-up of the patient. Too great reliance, however, should not be placed upon the biochemical tests ; Cahn (1948) points out that these may be normal if the disease is localized to one small area, for example, the maxilla.

In the five cases in the present series in which bone was examined, there was little difficulty in arriving at a histological diagnosis. Jaffe (1933) has pointed out, however, that the bone of the jaws contains more cement lines than does bone from other parts, and that these are often irregularly disposed, presumably because the stresses of mastication occasion a very active turnover of bone. He also mentions healing infectious periostitis with excessive bone formation as a possible cause of numerous cement lines and generalized osteitis fibrosa cystica as a cause of chaotic bony architecture. But when mosaics appear in conditions other than Paget's disease their extent is limited and the cement lines tend to be more regular.

Where teeth only are available for examination, a histological diagnosis may still be made with confidence when the cementum shows the changes just described. Curvilinear markings of that type are not found in cementum proliferation in any condition other than Paget's disease. Rushton's (1938) cases showed similar cemental changes and Thoma's (1950) cases probably do too, so far as can be judged from his illustrations. The point to which Thoma draws attention, however, is the extensive resorption of both cementum and dentine in his specimens, and the growth into the excavated area of trabeculae of typical Paget bone.

Summary

Paget's disease of bone may affect the jaws and teeth, sometimes as part of a more general affection of the skull or skeleton, sometimes as a solitary lesion. The microscopic appearances of the teeth and alveolar bone are described and the diagnosis discussed.

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ACUTE DISSEMINATED TORULOSIS ASSOCIATED WITH HODGKIN'S DISEASE

BY

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It is now generally agreed that organisms, such as diphtheroid bacilli, avian tubercle bacilli, and *Brucella*, are secondary invaders or contaminants in Hodgkin's disease. Since certain fungi may cause granulomatous lesions which somewhat resemble Hodgkin's disease, attempts have also been made to incriminate members of this group. In particular, attention has been paid to the yeast-like organism *Cryptococcus neoformans*. About 18 cases have been described in which a condition resembling Hodgkin's disease was associated with active torulosis, considered by some authors as the aetiological agent.

The purpose of this paper is to report a further case in which it was clear, from a study of the lesions at necropsy, that the torulosis was a terminal event in the course of advanced typical Hodgkin's disease.

Case Report

A housewife, aged 59, was admitted to hospital in June, 1953, for treatment of leg ulcers which had recurred many times during the previous eight years. The Wassermann reaction had been found to be positive and the ulcers were suspected of being gummatous. Bacteriological examination showed *Staphylococcus aureus* on two occasions, *B. proteus* on two occasions, and a diphtheroid once. No yeast-like organisms were ever obtained from the lesions by microscopic or cultural examination. The ulcers were treated by rest in bed, injections of penicillin, and the local application of saline packs. They healed successfully within the following three months.

At the beginning of August she developed a cough and signs of basal congestion of the lungs. At this time the liver was grossly enlarged with a nodular surface. The tip of the spleen was also palpable. Her temperature rose to 100.4° F. on August 5 and continued to rise daily to levels of 100 to 101.6° F. from morning temperatures of 97 to 98° F., in spite of the administration of penicillin and sulphamezathine. On September 7 her temperature became normal following the administration of aureomycin for one week, and it remained normal until her death. Blood culture performed on August 31 remained sterile after 11 days' incubation.

Liver function tests performed on August 28 were as follows:

Serum bilirubin, 0.95 mg./100 ml.; serum alkaline phosphatase, 16.2 units/100 ml.; thymol turbidity, over 10 units; thymol flocculation, + + +; and colloidal gold, 5 units.

On September 23 she was noted to be rapidly developing an ascites. This became very severe, and on October 13 paracentesis abdominis was performed. Examination of the centrifuged deposit of the ascitic fluid showed clumps of cells with darkly stained nuclei and scanty cytoplasm, which were interpreted as malignant cells. There were also large numbers of encapsulated yeast-like organisms, which grew on blood agar. The same organism was isolated from blood culture performed on October 14. Both were shown to be *Cryptococcus neoformans*.

The ascitic fluid rapidly reaccumulated. The patient became very drowsy and died on October 17.

The antibiotic therapy that she received during her last four months of life is listed in the Table, as it is thought to bear some relation to the behaviour of the cryptococcal infection.

TABLE

DOSAGE OF ANTIBIOTICS AND SULPHONAMIDES ADMINISTERED DURING LAST FOUR MONTHS OF LIFE

Antibiotic or Sulphonamide	Dates (inclusive)	Daily Dosage	Total Dosage
Penicillin (Distaquaine)	June 22–June 29 ..	0·6 million units	27·4 million units
	August 7–August 10 ..		
	August 26–September 1		
Soluble penicillin	October 2–17	1 million units	
Sulphamezathine	August 10–15	2 g.	12 g.
Aureomycin ..	September 1–8 ..	2 g.	18 g.
Streptomycin ..	October 2–17	2 g.	32 g.

Necropsy

Necropsy was performed 58 hours after death, the body having been kept at refrigerator temperature for most of that time.

The body was that of a thin, jaundiced woman. The peritoneal cavity contained a very large quantity of cloudy, yellow fluid.

There were enlarged lymph nodes in the para-aortic region, the hilum of the liver, the transverse mesocolon, and the pelvis. The lymph nodes were discrete, rubbery, and had a smooth surface (Fig. 1). The cut surface showed replacement of the greater part of each lymph node by homogeneous, greyish-white tissue, flecked with haemorrhagic areas.

The liver weighed 1,700 g. and was moderately enlarged (Fig. 1). The whole of its surface was covered with yellow nodules ranging from 2 mm. to 1 cm. in diameter. It was hard and difficult to cut. The cut surface showed yellowish-grey nodules, ½ to 1 cm. in diameter, in all parts. Between the larger nodules were smaller, greenish foci, 1–2 mm. in diameter. There was no obstruction of the extrahepatic biliary passages. The portal vein was patent.

The spleen weighed 340 g. It had a slightly irregular surface with numerous small greyish nodules projecting from a mottled, purple background. On section most of the splenic substance was replaced by firm white tumour, intersected by red streaks (Fig. 2). There was no gross abnormality of the lungs, brain, or meninges.

Histology

The greenish foci in the liver were islands of liver cells, often showing fatty degeneration, surrounded by mature fibrous tissues containing lymphocytes, plasma cells, and bile ducts (Fig. 3). The large liver nodules were islands of characteristic Hodgkin infiltration. They were composed of enlarged reticulum cells with occasional mitoses, numerous multinucleate cells of the Dorothy Reed variety, lymphocytes, and fibroblasts. No spirochaetes were found by Levaditi's method.

The spleen and enlarged lymph nodes were infiltrated by Hodgkin's tissue similar to that in the liver, with, in addition, areas of necrosis.

Histological examination of each lung showed emphysematous changes. Lying loosely within each alveolus were from one to five completely vacuolated round bodies with a brownish rim. They varied in size from 15 to 30 μ, and many of them were engulfed by histiocytes. Examination of a frozen section of lung by the Sudan IV stain showed that they were globules of fat. The Schultz reaction was negative, indicating that they

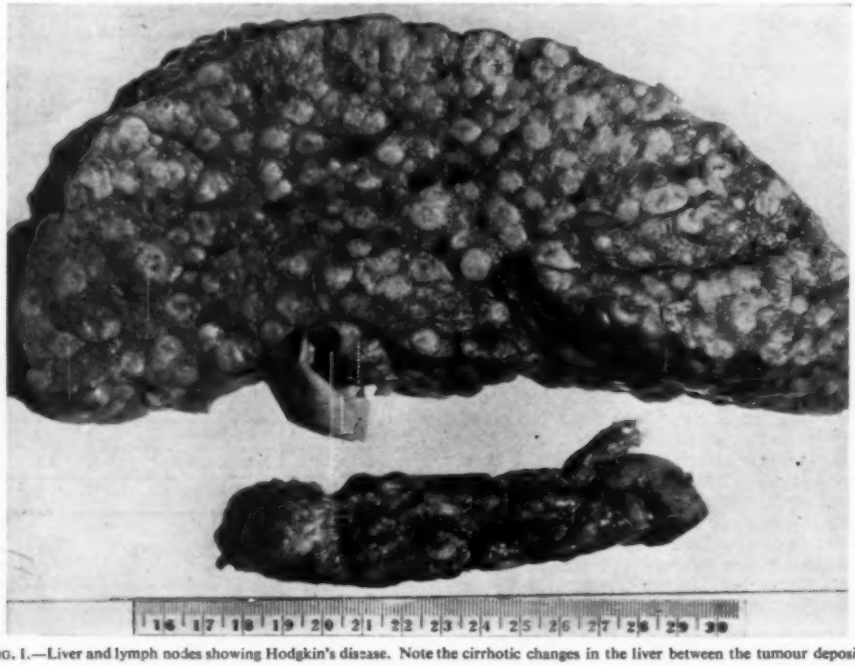

FIG. 1.—Liver and lymph nodes showing Hodgkin's disease. Note the cirrhotic changes in the liver between the tumour deposits.

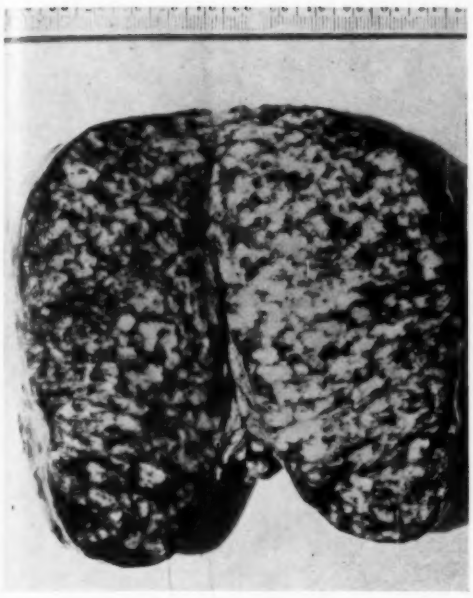

FIG. 2.—Spleen, showing almost complete replacement by Hodgkin's tissue.

did not contain cholesterol. Their presence might be interpreted as the aftermath of a pneumonia, of which there was some clinical evidence about 10 weeks before death, but the appearance of the fat as large free globules is against this and rather suggests that it was derived by aspiration. There is, however, no clinical evidence in support of this latter view.

Small collections of histiocytes were found throughout the lungs at a density of about one group to every two low-power fields. They were situated in the lumen of the alveolus close to the alveolar wall, and were often in the vicinity of a small vessel. Cryptococci were later demonstrated within these cell groups, but could not be seen in the ordinary haematoxylin-and-eosin preparations.

The cryptococci were clearly seen when smears of the deposit of ascitic fluid were stained with haematoxylin and eosin or by the P.A.S. (McManus) technique. When these stains were used on histological section of the viscera, however, the organisms could be seen only with difficulty or not at all. It was found that staining sections with haematoxylin and mucicarmine clearly demonstrated them, by staining the mucinous cell wall a deep red. Making use of this stain, most of the organs were examined for cryptococci and their lesions.

In all areas of the lungs small numbers of encapsulated cryptococci were found within the clusters of histiocytes described above. Some of the organisms occupied an intracellular position. In the adrenals occasional histiocytic collections were adjacent to the walls of the veins, and these, likewise, contained small numbers of cryptococci (Fig. 4).

Section of the brain and meninges in the region of the motor cortex showed minute lesions composed of histiocytes and cryptococci lining the subarachnoid space on both the pial and arachnoid surfaces.

The pars intermedia of the pituitary contained moderate numbers of organisms, but very little histiocytic reaction.

Groups of cryptococci were found in the following organs, associated occasionally with areas of necrosis, but with no surrounding cellular reaction: in some of the connective tissue septa of the pancreas, in occasional glomerular capillaries of both kidneys, in the thyroid, in the sinuses of

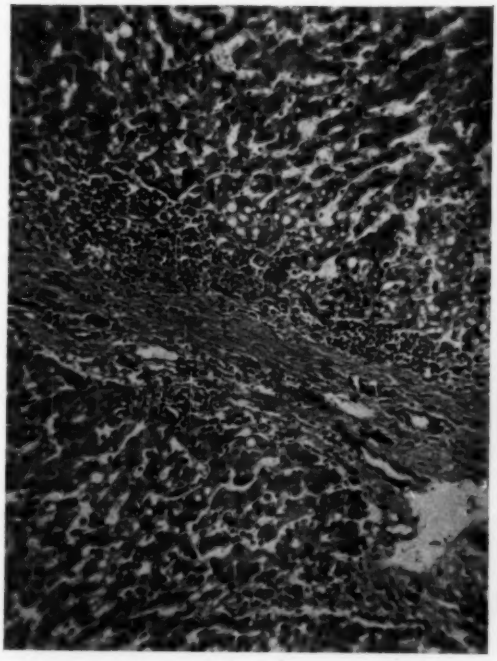

FIG. 3.—Liver, showing fatty change and a band of fibrous tissue associated with lymphocytes. (Haematoxylin and eosin. ×100.)

some abdominal lymph-nodes, and in the spleen. No cryptococci were found in single sections taken from two blocks of the liver.

Mycology

The organisms were round and varied from about 3 to 8 μ in diameter. Occasional budding forms were present. In the tissues and in the ascitic fluid the cryptococci were surrounded by a wide capsule which was not stained in any of the staining procedures that were used (Fig. 5). The capsule was not present when the organism was grown in culture. The periphery of the organism stained a deep red by P.A.S. and mucicarmine, while the inner part stained pink by these stains.

The organism grew easily under aerobic conditions on most of the usual laboratory media. After 48 hours' incubation on horse blood agar at 37° C. it appeared as brownish-yellow, raised, round colonies about 0.5 mm. in diameter. Growth was slow at room temperature. No gas was produced when the organism was grown in glucose, saccharose, lactose, mannitol, galactose, or dulcitol.

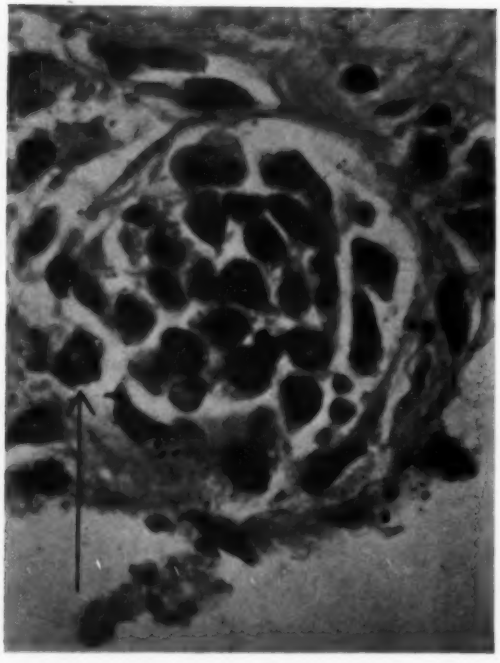

FIG. 4.—Edge of vein in adrenal showing a clump of histiocytes with a single intracellular cryptococcus (marked by arrow) and staining red in section. (Haematoxylin and mucicarmine. ×900.)

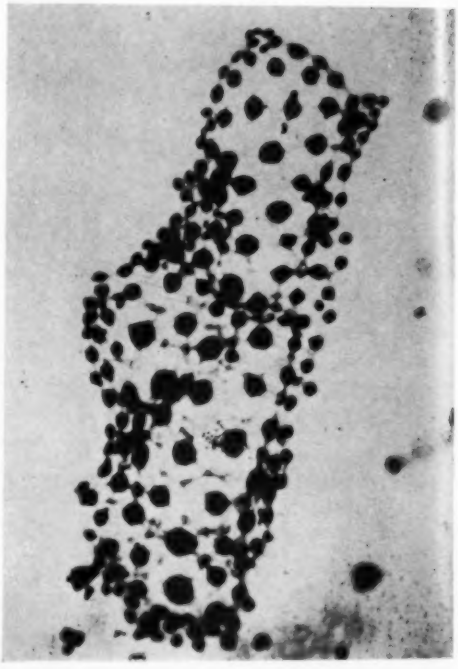

FIG. 5.—Clump of cryptococci from ascitic fluid, showing the wide unstained capsule around each organism. (P.A.S. ×450).

No mycelium was found in the tissues or by prolonged growth of the organism in corn-meal agar at room temperature. Ascospores were not seen when the organism was grown on carrot-gypsum slopes. Intraperitoneal injection of saline suspensions of cryptococci from culture into mice resulted in death in from five to six weeks after inoculation. Large numbers of cryptococci were found in the viscera of the mice. The complete mycological identification of this organism as the pathogenic form of *Cryptococcus neoformans* was performed by Dr. J. Walker, of the Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine.

Discussion

It is very unusual for Hodgkin's disease to present clinically with liver failure as the predominant feature. Fairbank (1953) describes three such cases and states that there are no previous reports in the literature. In two of his cases the liver was diffusely involved by Hodgkin's deposits, but there is no mention of cirrhotic changes. In the case described here the acute liver failure was

due to the remarkable combination of widespread Hodgkin's lesions in the liver with portal cirrhosis.

The association of cirrhosis with Hodgkin's disease of the liver must be very rare, since mention of only one comparable case was found in the world literature (Steiner, 1936).

It is possible, therefore, that the cirrhosis was pre-existing. Hodgkin's disease developing in such a liver might produce a much more widespread growth than usual.

It would seem equally possible that the cirrhotic lesions arose as a result of long-standing diffuse Hodgkin's disease involving the liver. Small focal necroses of areas of liver unaffected by the Hodgkin's lesions are said to be common in Hodgkin's disease and result, perhaps, from an interference with the lobular blood supply due to giant-cell embolism (Pullinger, 1932). It is possible that a slower vascular interference might cause, not necrosis, but fibrosis. It should be stressed, however, that apart from the cirrhotic changes in the liver the histological features of the liver, spleen, and lymph nodes were typical of Hodgkin's disease.

Infection with *Cryptococcus neoformans*, in the great majority of cases, mainly involves the central nervous system, either as a diffuse meningitis or as a granulomatous lesion of the brain. In this case there were no neurological symptoms or signs and, although an early cryptococcal meningitis was found histologically, it was but a small part of a disseminated infection, the brunt of which was suffered by the lungs and ascitic fluid. So great were the numbers of organisms found in the latter that it is not surprising that there was a positive blood culture, which is uncommon in cases of torulosis.

The fact that the cryptococcal disease took the unusual form of an overwhelming, acute infection may be related in this case to the heavy antibiotic therapy which the patient received in the last three months of her life. It is of interest that Symmers' second patient (1953), who received penicillin, streptomycin, aureomycin, and chloromycetin within the last six weeks of life, also had a fulminating infection. It is not yet known whether antibiotics directly stimulate the multiplication of *Cryptococcus neoformans* as occurs with *Candida albicans* (Foley and Winter, 1949).

It would appear to be established that there is some relationship between Hodgkin's disease and torulosis, since the number of described cases showing association of the two diseases is too great to be accidental. Gendel, Ende, and Norman (1950) found 13 cases in the literature and described one of their own, a total of 8.5% of all the then reported cases of torulosis. Since then four further cases have been described (Rodger, Terry, and Binford, 1951; Collins, Gellhorn, and Trimble, 1951; Holmes and Hawks, 1953; Symmers, 1953). Moreover, Gordon (1932) isolated a form of this organism from the lymph nodes of two cases of Hodgkin's disease, while Collins *et al.* (1951) obtained it by gastric aspiration in a case of Hodgkin's disease showing no clinical evidence of cryptococcal infection.

The nature of the association is still in doubt. In our case there is strong evidence that the cryptococci arose as a terminal infection in a case of typical Hodgkin's disease. The acute character of the infection is in sharp contrast with the advanced state of development of the Hodgkin's lesions. Moreover, most sections of the neoplastic areas showed no cryptococci when stained with mucicarmine. Where cryptococci were seen in proximity to the lesions it was clear that their presence was incidental, as in the abdominal lymph nodes in which yeasts were found in the peripheral sinuses but not in the adjacent neoplastic tissue.

Some authors consider, however, that the Hodgkin's lesions in their cases were the result of infection with cryptococci. Symmers (1953), for instance, regards the Hodgkin's lesions in his first case as an atypical granulomatous reaction to the fungus infection. Yet his case, with some of the other cases where a similar claim is made, may be equally interpreted as Hodgkin's disease complicated by cryptococcal infection. The presence of cryptococci in the original biopsy material taken three years before death is not conclusive proof, in Symmers' case, that cryptococci initiated the Hodgkin's lesions. The latter were typical of lymphadenoma in their histological features, their distribution, and, in the case of the superficial lymph nodes, in their reaction to irradiation. It is difficult to accept that an organism can produce granulomatous lesions only in the brain and skin while stimulating the development of reticulosarcoma-like changes elsewhere in the body. It is possible that the presence of the fungi in the neoplastic lesions was accidental. At necropsy of Symmers' case they were scanty in the Hodgkin's tissue and absent from the reticulosarcoma, but they were easily found in the granulomatous lesions of the brain and skin. In our case the presence of cryptococci in any organ of the body can be accounted for by the septicaemia, and this may have been present at some stage of the disease in Symmers' case.

The explanation for the association between the two diseases may be that cases of Hodgkin's disease

are peculiarly susceptible to torular infection, owing to a derangement of immunity mechanisms. It has been shown that an anergic state to intracutaneous tuberculin develops in the course of Hodgkin's disease in the majority of cases (Reed, 1902 ; Hoffmann and Rottino, 1950). Dubin (1947) also found that the incidence of positive serological reactions for syphilis was lower in Negro cases with Hodgkin's disease than in the Negro population in general. It may be that in Hodgkin's disease there occurs, likewise, a deficiency of antibody normally providing defence against cryptococci.

It is probable that some cases of torulosis escape diagnosis both during life and at necropsy. Attention was drawn to the presence of a cryptococcal infection in this case only just before death when the organisms were isolated from the ascitic fluid and the blood. The patient at this time showed no pyrexia or any other features that indicated that she was suffering an overwhelming infection. At necropsy there was no gross evidence of infection and no fungi were seen on microscopical examination of tissue sections stained by the usual methods. Only when, on the advice of a mycologist, sections were stained with haematoxylin and mucicarmine was it possible to identify cryptococci in the tissues. We would, therefore, recommend the routine use of this stain in the histological examination of pathological material for yeasts. The diagnosis of torulosis should be especially considered in the investigation of cases of Hodgkin's disease.

Summary

A case of Hodgkin's disease complicated by torulosis is described.

The clinical features were those of acute hepatic failure. At necropsy it was found that multiple Hodgkin's lesions in the liver were associated with portal cirrhosis.

Cryptococcus neoformans was isolated from the blood and ascitic fluid shortly before death. The torular lesions found at necropsy were microscopic, disseminated collections of histiocytes and cryptococci.

The relationship between Hodgkin's disease and torulosis is discussed. The possibility of heavy antibiotic therapy stimulating the cryptococcal infection is mentioned.

We wish to thank Dr. J. Davson, Group Pathologist, North Manchester Group of Hospitals, for his help and advice, and Dr. R. W. Luxton, for permission to publish this case, which was under his care at Crumpsall Hospital. We are grateful to Dr. J. Walker, of the Mycological Reference Laboratory, for full identification of the yeast, and to Miss J. Perry for the photographs.

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J. clin. Path. (1955), 8, 207.

TORULOSIS ASSOCIATED WITH HODGKIN'S DISEASE

BY

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Infections due to *Cryptococcus neoformans*, a pathogenic yeast causing the disease torulosis, have been reported infrequently in Britain, but are more common than the 10 cases so far published would suggest (Symmers, 1953). Torulosis is a world-wide disease and is endemic in Britain. The association of this infection with Hodgkin's disease has been reported previously (Gendel, Ende, and Norman, 1950), but its apparent rarity in Britain is the reason for presenting an example of recent occurrence.

Case History

A 41-year-old man, a cardboard blocker by trade, was admitted to the Prince of Wales Hospital, London, on December 1, 1949, complaining of weakness and loss of energy for three months. Just before admission he had noticed swelling of the ankles, and had developed an unproductive cough. His previous health had been good and his weight stationary. He had not resided outside the United Kingdom, apart from two years of Army service in north-west Europe, since 1944.

On examination he was found to be a normally developed man, although somewhat spare and pale. His temperature and pulse rate were normal. His tongue was smooth. There was some abdominal distension and the liver was palpable over three finger-breadths below the costal margin. The spleen could not be felt, nor any enlarged lymphatic glands. The urine contained a trace of albumin. There was a normochromic anaemia (Hb 9.4 g.%) and a leukopenia (1,300 white cells per c.mm., of which 56% were polymorphs, many of them young cells). Sternal puncture showed active normal marrow. Serum was low in total protein (5.4 g.%) with a normal albumin-globulin ratio, high alkaline phosphatase (33 units—Jenner and Kay), and normal flocculation reactions (colloidal gold, thymol turbidity, and Takata Ara tests). The erythrocyte sedimentation reaction was within normal limits. The Paul-Bunnell test and the Wassermann reaction were negative. *X*-ray examinations of the chest, alimentary tract, and skeletal system were normal. The temperature, which at first showed small irregular elevations up to 100° F. (37.8° C.), gradually became remittent. Repeated blood cultures incubated for six weeks were sterile, and Widal and brucella agglutination tests were negative. There was no response to treatment with penicillin, streptomycin, vitamin B_{12}, or proteolized liver. Only blood transfusions brought about clinical improvement, but this was temporary.

After three months' observation, the spleen became palpable, and small discrete rubbery glands were noted in the right groin, one of which was removed for histological examination. This was a small, grey, soft lymph node almost completely replaced by a follicular granulomatous reaction, superficially resembling tuberculosis (Fig. 1). The follicles were composed of endothelioid cells, numerous giant cells, and lymphocytes and plasma cells. In many of the follicles there were round yeast-like bodies, staining weakly with haematoxylin, and surrounded by clear spaces; they resembled *Cryptococcus neoformans*. The concentration of the yeast varied in different parts of the tissue from occasional cells to large aggregations, and were sometimes situated within giant cells (Fig. 2). The granulomatous reaction extended into the fatty connective tissue around the gland, and involved one small artery, projecting into its lumen (Fig. 3).

Two months later another lymph node enlarged at the same site as the first, and on this occasion fungus species identification was successful by culture of the biopsy specimen. At this time, too, a crusting ulcer, about 1 cm. in diameter, developed on the lower lip. Scrapings from this contained yeasts with hyphae and Gram-positive cocci; *Candida albicans* only was isolated in cultures. The lesion healed slowly without scarring when painted with gentian violet.

The patient was treated successively with potassium iodide, streptomycin, penicillin, aureomycin, and chloromycetin, but these failed to bring about any clinical improvement. Intravenous gentian violet was given but abandoned when it produced venous thrombosis. Sulphadiazine, however, appeared to reduce the pyrexia and produce both subjective and objective improvement. It produced a gain in weight of 6 lb. in three weeks, and the patient was able to get up again after several months in bed. Sulphadiazine was discontinued after 75 g. had been given, but this was followed by deterioration in his condition. Further courses of sulphonamides were given with the same good response, but had to be abandoned when the total white cell count fell below 800 per c.mm.; a total of 645 g. of sulphonamide drugs was given. The

FIG. 1.—Section of a lymph node from the groin showing a follicular granulomatous reaction and encapsulated yeasts. Stained by haematoxylin and eosin (× 58).

general condition continued to deteriorate apart from one short spontaneous remission, and the patient died on October 10, 1950, 13 months after the onset of symptoms.

Skin tests carried out with a suspension of a strain of *Cryptococcus neoformans* isolated from the lymph node were negative. Histoplasmin, 1 in 100, coccidioidin, 1 in 1,000, and blastomycin, 1 in 1,000, also gave negative results. No complement-fixing antibodies were detected in the patient's serum when the homologous *Cryptococcus neoformans* suspension was used as antigen. Attempts to isolate *Cryptococcus neoformans* from the blood and the alimentary, respiratory, and urinary tracts were unsuccessful.

Post-mortem examination showed general wasting, a grossly enlarged liver (2,250 g.) and spleen (900 g.). These contained scattered areas of greyish infiltration surrounded by congestion. The para-aortic abdominal and posterior mediastinal lymph nodes were also enlarged. No abnormalities were found in the brain or spinal cord, lungs, heart, alimentary tract, kidneys, or endocrine glands. Mid-femoral bone marrow was red and hyperactive.

Histologically, Hodgkin's disease was present in the liver, spleen, and para-aortic glands. Normal tissue was replaced by lymphocytes intermingled with reticulum cells having large multiple or convoluted nuclei typical of "Sternberg Reed" cells (Fig. 4). No cryptococci were found in these areas after prolonged search using special strains. The nodes from the groin in the region of the biopsies showed fibrosis only. *Cryptococcus neoformans* was not grown in cultures made from the liver, spleen, lung, brain, and abdominal lymph nodes.

Mycological Investigations.—In tissues, the yeasts were surrounded by wide capsules, best demonstrated in smears by India ink or nigrosin, and staining easily with stains for mucopolysaccharides (periodic-acid-Schiff method, toluidine blue, and mucicarmine). The *Cryptococcus neoformans* culture isolated from the gland biopsy was cream-coloured at first and became brownish and slimy later. Microscopy showed yeasts producing single blastospores but no hyphae; abortive hyphae were found only in the tissues. The cells were weakly Gram-positive, spherical and 3–10 μ in diameter, with double contoured cell walls seen as highly refractile rings in wet preparations. Capsule formation was noted in old cultures.

On blood agar growth at 37° C. was visible after two days and reached maximal size, 2–3 mm., in two weeks. On Sabouraud's agar at 37° C. growth was more rapid and was so mucoid that cultures flowed to dependent parts of the slopes. The yeast was relatively inert when submitted to fermentation tests; acid was produced without gas in peptone water containing 1% glucose and saccharose, but there was no fermentation of other sugars (lactose, maltose, dulcite, man-

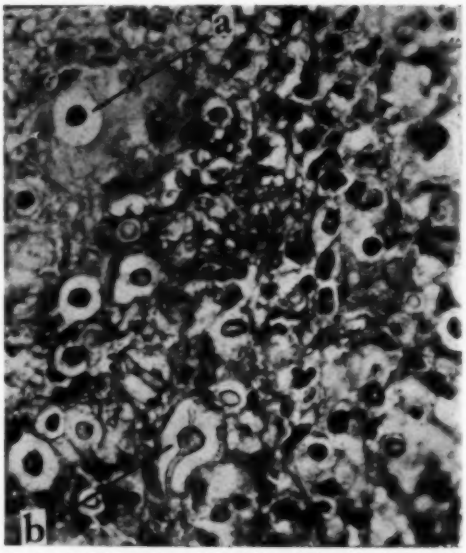

FIG. 2.—Section from the centre of a large follicle showing yeast-like organisms surrounded by clear zones. One cryptococcus (a) lies within a giant cell; another (b) shows abortive hypha formation. Stained by Gram-carmalum. (× 600).

FIG. 3.—Section showing a small artery near the node illustrated in Fig. 1; intimal follicles are seen bulging into its lumen. Stained by haematoxylin and eosin. × 58).

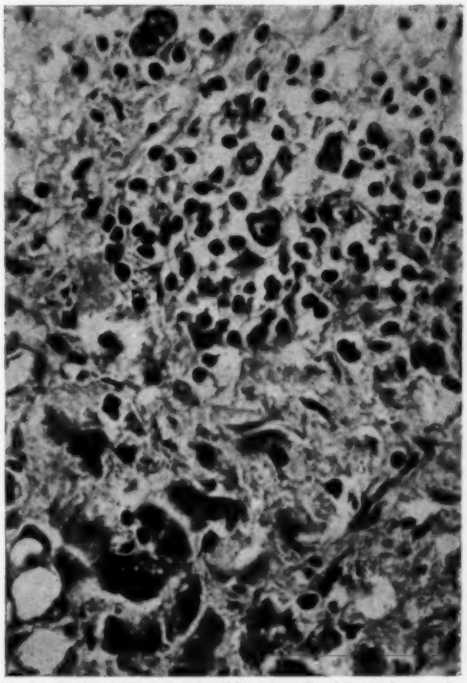

FIG. 4.—Section of liver showing "Sternberg-Reed" cells in granulomatous infiltration. Stained by haematoxylin and eosin. (× 600.)

nite, xylose, arabinose, inositol, dextrin, and salicin). Gelatin was not liquefied, and no hyphae were formed in corn meal agar.

Sensitivity tests showed no inhibition of growth by sulphonamides in concentrations of 20 mg.% of medium, nor by penicillin, chloromycetin, terramycin, aureomycin, or streptomycin at concentrations 10 times those obtainable in the body. Inhibition of growth by gentian violet occurred at a concentration of 1 in 10,000.

The culture was virulent for mice. Three mice injected intraperitoneally with 0.2 ml. of a cell suspension from a young culture died between five and seven weeks with gelatinous nodules containing masses of cryptococci in the lungs, abdomen, and brain.

Discussion

The finding of *Cryptococcus neoformans* in the soil (Emmons, 1951) brings torulosis into line with other systemic mycoses, such as histoplasmosis, coccidioidomycosis, and aspergillosis, in which the causative organisms occur as saprophytes in nature. *Cryptococcus neoformans* is of low infectivity and human transmission of disease has not been reported.

In their exhaustive review of torulosis, Cox and Tolhurst (1946) note that roughly 8% of the reported cases were associated with Hodgkin's disease. It is suggested that prolonged nutritional deficiency in Hodgkin's disease is a factor in producing this striking association, for it is well known that in debilitated states non-pathogenic organisms and fungi may overcome the normal body defences and become invasive. In support of this, the work of Benham (1935) indicated that non-pathogenic strains of *Cryptococcus neoformans* would infect rats fed on a vitamin-deficient diet. There has been no convincing evidence that infection by this yeast can produce Hodgkin's disease. In the present case it is believed that torulosis was superimposed on a pre-existing lymphadenopathy. The infection was localized to the granulomatous lymph nodes of the right groin as far as could be established by histological and bacteriological investigations; no cryptococci were found in other regions affected by Hodgkin's disease. The presence of *Candida albicans* in the ulcer of the lip and in the sputum and faeces of

this patient is considered to be without significance. In retrospect, the anaemia, leukopenia, pyrexia, and progressive debility are attributed to Hodgkin's disease and not to torulosis. It is unusual in torulosis for the brain and lungs to escape involvement as apparently occurred in this man, but the rapid progress of the reticulosis may have forestalled this. Evidence of primary infection was not found at necropsy. Treatment of torulosis is still empirical, for although sulphonamides in large and prolonged dosage are often temporarily successful the remissions so characteristic of this disease make evaluation of their efficacy impossible.

Summary

Infection with *Cryptococcus neoformans* in a man suffering from Hodgkin's disease is described. The mycological investigations are presented and the interrelation between torulosis and Hodgkin's disease is briefly discussed.

I wish to thank Dr. Bruce Williamson for permission to use the clinical record, Dr. L. M. Franks for putting at my disposal the post-mortem material, and Dr. R. W. Riddell for his advice and encouragement.

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A CASE OF PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA ASSOCIATED WITH SECONDARY HAEMOCHROMATOSIS, A LOWER NEPHRON NEPHROSIS, AND A MEGALOBLASTIC ANAEMIA

BY

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Secondary or exogenous haemochromatosis has been defined by Aufderheide, Horns, and Goldish (1953) as a condition acquired as "a consequence of anaemia, blood transfusion, or both, and characterized by increased hepatic and total body iron and unequivocal portal cirrhosis." These authors found only 20 cases in the literature and have added two more of their own. They comment on the lack of adequate evidence of true portal cirrhosis in many of the cases described.

The association of paroxysmal nocturnal haemoglobinuria (P.N.H.) and secondary haemochromatosis has not been previously described. Each of these conditions produces characteristic but totally dissimilar patterns of iron distribution in the body. In classical paroxysmal nocturnal haemoglobinuria renal haemosiderosis is associated with depletion of the normal iron depots, while in secondary, as in primary, haemochromatosis the body tissues are saturated with iron but the kidney contains little or none. The iron distribution in these two conditions represents the results of opposing processes. In paroxysmal nocturnal haemoglobinuria there is a constant daily loss of iron, whereas in secondary haemochromatosis iron is accumulated in the body tissues.

In this paper we propose to discuss the pathogenesis of this unusual and paradoxical combination of iron distribution.

The occurrence of a fatal lower nephron nephrosis and the presence of a megaloblastic anaemia lend further interest to the case.

Clinical Data

The patient, a housewife aged 44 years, was first seen in June, 1949. She was admitted to hospital with the signs and symptoms of a severe anaemia. There was an associated history of menorrhagia and bleeding haemorrhoids. The skin had a faint icteric tinge and the liver edge was easily palpable. The initial blood count was: Hb, 1.7 g. per 100 ml.; red cells, 545,000 per c.mm.; colour index, 1.1; white cells, 4,000 per c.mm.; and platelets, 51,000 per c.mm. The red cell fragility test was normal. Sternal puncture showed a normoblastic hyperplasia. The Wassermann reaction was negative. The serum bilirubin level was 0.8 mg. per 100 ml. and the remaining liver function tests were normal. Following transfusion with 2 pints of blood, and the administration of "ferrivenin" and oral iron, the haemoglobin rose to 10 g. per 100 ml., and the patient was discharged with instructions to continue oral iron at home.

In September, 1950, she was readmitted with a recurrence of the symptoms of anaemia and an exacerbation of the menorrhagia. The Hb was now 4.5 g. per 100 ml. The liver was still enlarged and a second sternal marrow puncture showed that erythropoiesis was again normoblastic. Two pints of blood were given, and a tinge of jaundice present on admission became more obvious. The serum bilirubin level was 2.5 mg. per 100 ml., the serum alkaline phosphatase 22 King–Armstrong units per 100 ml., thymol turbidity 14 units, thymol flocculation 4+, and serum colloidal gold 5. Treatment with "ferrivenin" and oral iron restored the haemoglobin to 10 g. per 100 ml. A hysterectomy was then performed. Shortly afterwards she was sent home and instructed to continue taking oral iron.

In August, 1952, she was again readmitted with Hb 3.5 g. per 100 ml., colour index 1.1, and a reticulocyte count of 8%. The liver was still enlarged and the marrow again showed normoblastic erythropoiesis. Marked generalized brown skin pigmentation was noted for the first time. The liver function tests were as before and the serum proteins were normal. After treatment with a pint of packed cells followed by a course of oral iron she was discharged in September, 1952.

In January, 1953, she was readmitted for the fourth time with a recurrence of symptoms. The brown pigmentation of the skin had increased. The liver was still palpable. The Hb was 4.5 g. per 100 ml. and the

colour index 1.3. A sternal marrow puncture now showed megaloblastic erythropoiesis. A review of the previous marrow smears confirmed the absence of megalopoiesis in them. The stomach contained free acid and a four-day fat balance showed a 93% fat absorption. A diagnosis of megaloblastic anaemia associated with cirrhosis of the liver was entertained at this stage. Despite a course of intramuscular vitamin B_{12} and a crude liver extract, the marrow remained predominantly megaloblastic. A course of folic acid, however, produced a reticulocyte response of 36% and erythropoiesis was now normoblastic. A glucose tolerance test was normal.

Haemoglobinuria was first noted during this period of admission. When questioned, the patient admitted having passed dark urine on many occasions during the previous three or four years. Oxyhaemoglobin was detected spectroscopically in the urine and was present in the absence of whole red blood cells. Haemosiderinuria was also noted. The direct and indirect Coombs tests were negative. No cold agglutinins or cold or warm haemolysins could be demonstrated. There was no increase in the red cell osmotic fragility. The Ham test (Ham, 1939) and Crosby's modification of this test (Crosby, 1950) were positive. The Hegglin and Maier (Hegglin and Maier, 1944) and Schumm's tests were positive. On the basis of these findings, a diagnosis of paroxysmal nocturnal haemoglobinuria was made. The patient was discharged in May, 1953.

In February, 1954, the patient was admitted for the last time. She had been having attacks of vomiting attributed by herself to the taking of iron tablets. The Hb was 4.5 g. per 100 ml. and the colour index was 1.2. Anticoagulant therapy was begun and the prothrombin level maintained between 15 and 25% of average normal for a fortnight. No apparent improvement followed, and it was decided to transfuse packed cells which had been washed three times. A severe reaction followed. The patient complained of severe abdominal pain and vomited two to three hours after transfusion. Laboratory tests, including the Coombs test, showed no incompatibility. Jaundice and oliguria followed, the blood urea rose rapidly to 320 mg. per 100 ml., anuria developed, and despite prompt institution of the Bull régime the patient died 10 days after transfusion.

Post-mortem Findings

A complete necropsy was performed five hours after death. The body was that of a fairly well nourished middle-aged woman, with diffuse bronze pigmentation of the entire skin. Many small, unpigmented acne scars were present on the face. A lower abdominal operation scar was noted. There was no caput medusae.

The heart was of average size and weighed 300 g. There was no apparent valvular or coronary artery disease. The myocardium was browner than normal. The left pleural cavity contained a small effusion. The left lung weighed 220 g. and the right 300 g. They were grossly normal. The liver weighed 1,800 g. and its lower border projected 4 cm. below the costal margin. Its surface showed a coarse irregular nodulation. The cut surface was a dark bronze. The gall bladder and biliary ducts showed no anomaly. The pancreas was a pale yellowish-brown. Grossly it appeared normal. The spleen weighed 200 g. Its cut surface was bronzed. Many of the abdominal lymph glands were enlarged and were of a rusty brown. The adrenals showed irregular brown pigmentation of the periphery of their cortices.

The kidneys were about equal in size ; the right weighed 120 g. and the left 110 g. Their capsules were thickened and when stripped left a granular surface. The cortices were regular but somewhat narrowed and showed deep bronze pigmentation, while the pyramids were a brownish-grey. The hilar fat was increased.

The cerebral vessels and meninges were normal. A few small petechial haemorrhages were present in the pons, otherwise the brain was normal.

Red bone marrow hyperplasia was indicated by the presence of red marrow occupying the entire shaft of the femur.

Chemical analysis of the liver showed a total iron content of 21 g.

Microscopic Findings

The skin showed marked brown pigmentation of the basal cells of the Malphigian layer. The pigment gave a negative Prussian-blue reaction, was bleached by hydrogen peroxide, and Fontana's stain for melanin was positive.

The liver exhibited a marked increase in the periportal connective tissue with intercommunicating portal areas and distortion of the hepatic architecture ; considerable bile-duct proliferation was present in the portal areas. The appearances were those of a portal cirrhosis. In addition there was a massive hepatic haemosiderosis with heavy deposits of haemosiderin in the liver cells, Kupffer cells, and portal connective tissue. The bile canaliculi contained only minute traces of stainable iron.

The pancreatic acini contained moderate scattered deposits of haemosiderin. The islets were little affected. Occasional foci of fibrosis were noted.

The spleen and lymph glands contained considerable haemosiderin deposits, these being concentrated mainly in the histiocytes and littoral cells of the dilated sinuses.

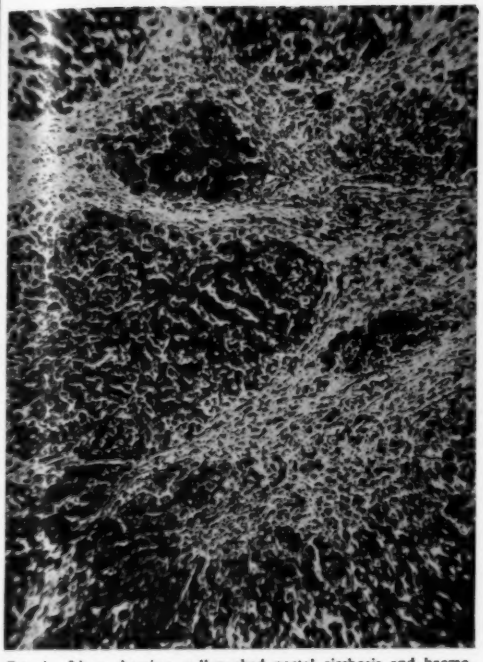

FIG. 1.—Liver showing well-marked portal cirrhosis and haemosiderosis.

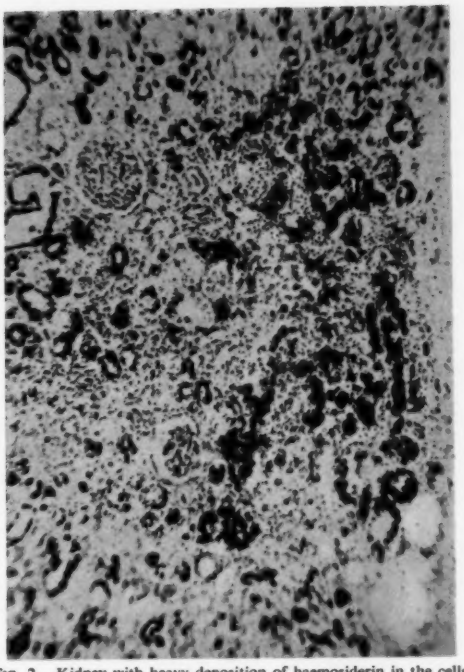

FIG. 2.—Kidney with heavy deposition of haemosiderin in the cells of the convoluted tubules.

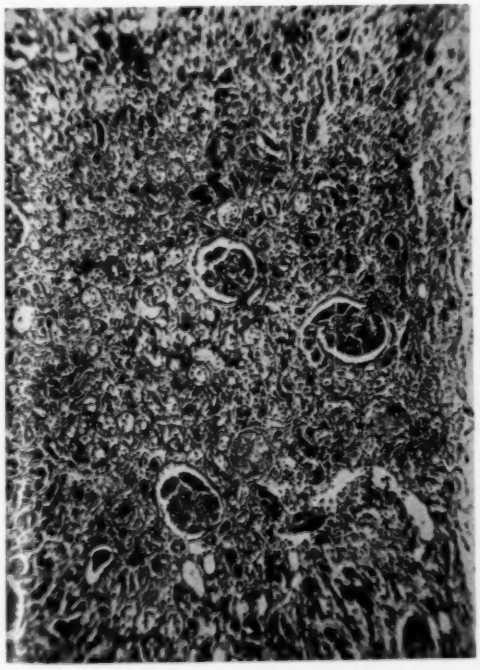

FIG. 3.—Kidney cortex, in which two fibrosed and three normal glomeruli are shown.

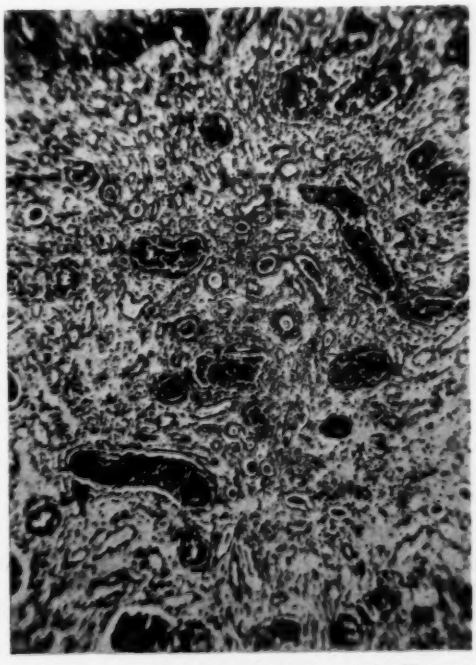

FIG. 4.—Renal medulla with many collecting tubules filled with haemoglobin casts.

The salivary glands contained small traces of haemosiderin. None was detected in the sweat glands.

The stomach mucosa appeared healthy and most of the glands contained haemosiderin.

The myocardial fibres contained small deposits of haemosiderin, situated at each pole of their nuclei in the position of the lipochrome of brown atrophy. Some fibres showed the presence of lipochrome without associated haemosiderin.

The muscle fibres of the tongue contained small traces of haemosiderin.

All smooth muscle appeared free of haemosiderin.

The lungs were emphysematous and showed minute scattered traces of haemosiderin, situated in phagocytes in the alveolar wall and free in the alveoli.

The pituitary contained small scattered deposits of haemosiderin, but there was no evidence of associated parenchymatous damage.

The thyroid was free of pigment.

The adrenal cortex contained considerable deposits of haemosiderin concentrated almost entirely in the glomerular zone.

The kidney's capsule was thickened and its cortex somewhat thinned. Large amounts of haemosiderin were present in the epithelial cells of the cortical tubules. Many tubules contained large fragments of haemosiderin lying free within their lumen. Some were dilated and showed degenerative and desquamative changes. Occasional small groups of subcortical tubules were atrophied and consisted of contracted tubules lined by a flattened epithelium with haemosiderin filling their small lumen. These groups were obviously not functioning and showed related chronic inflammatory cells. The glomeruli and arterioles contained no haemosiderin. A considerable number of fibrosed glomeruli were scattered throughout the cortex and there was a general increase of fine interstitial fibrous tissue. Most of the collecting tubules were distended by haemoglobin casts. Degenerative tubular cells were adherent to the casts, which were being attacked by polymorphs and macrophages. Foreign-body giant cells were seen in relation to some. Signs of regeneration were present in some tubules. These were lined and partly filled by a newly proliferated cubical epithelium. Scattered chronic inflammatory cells were present in the somewhat oedematous interstitial tissue of the medulla.

The final diagnosis included paroxysmal nocturnal haemoglobinuria, lower nephron nephrosis, and secondary haemochromatosis.

Discussion

Renal Damage and Paroxysmal Nocturnal Haemoglobinuria.—Crosby (1953) points out that the intense renal siderosis so characteristic of paroxysmal nocturnal haemoglobinuria does not, apparently, lead to impairment of renal function. When renal failure does occur it is generally a sequel of recurrent attacks of pyelonephritis. On the other hand, Heitzman, Campbell, and Stefanini (1953) described the development of a "haemosiderin nephrosis" in a case of paroxysmal nocturnal haemoglobinuria. During the course of their patient's illness a lower nephron nephrosis with anuria developed following transfusion with heparinized blood. Recovery followed after 14 days and the patient died nearly three years later from extrarenal causes. They discuss the relative parts played by the haemosiderosis and the lower nephron nephrosis in the production of the tubular degeneration and interstitial fibrosis which were such prominent features in their patient's kidneys. They concluded that the intense renal siderosis was responsible for the greater part of the renal damage and that the lower nephron nephrosis which had occurred three years previously contributed only slightly to the final renal pathology.

In our case there is also evidence of renal damage produced by haemosiderin deposition, although not to the same extent found by Heitzman. The fibrosed glomeruli, the contracted and atrophied tubules filled with haemosiderin, and the fine interstitial fibrosis can reasonably be attributed to the long-standing accumulation of haemosiderin, and not to the lower nephron nephrosis, which was only of 10 days' duration. Nissim (1953), by administering large doses of parenteral saccharated iron oxide to animals, has produced renal damage consisting of cloudy swelling and nuclear degeneration of the cells of the proximal convoluted tubules. Some cellular infiltration was also present around the degenerated tubules and intertubular foci of iron-laden phagocytes was also noted. It would thus appear that severe renal siderosis may produce a varying degree of kidney damage.

Anuria in Paroxysmal Nocturnal Haemoglobinuria.—The development of a fatal lower nephron nephrosis appears to be extremely uncommon. Crosby (1953) lists the causes of death in 53 cases, and lower nephron nephrosis is not mentioned amongst them. The rarity of a fatal lower nephron nephrosis is rather surprising, since almost all these patients receive numerous transfusions which are frequently followed by haemolytic crises

of varying severity. Haemolysis here is due to the presence in normal, fresh plasma of a heat-labile haemolytic factor to which the cells of patients with paroxysmal nocturnal haemoglobinuria are unduly sensitive, thus causing haemolysis of the patient's own cells and not those of the donor (Dameshek and Neber, 1950). The heat-labile factor responsible for this "plasma transfusion reaction" deteriorates on storage. If the red cells are freed from plasma by adequate washing with saline the reaction will not occur. Patients vary greatly in their sensitivity to this heat-labile factor. Crosby and Stefanini (1952) describe a case in which the cells had to be washed six times before they could be transfused with safety.

In our case mild haemolytic reactions followed the use of whole blood. The severe and ultimately fatal reaction followed the use of thrice-washed packed cells. This may be due to the fact that in the case of the earlier transfusions the whole blood had been stored for several days before use, while the washed cells used for the final transfusion were prepared from freshly collected blood and given without delay. The heat-labile factor had probably deteriorated in the stored blood whereas the washed cells might have still retained traces of fresh plasma in which the heat-labile factor was active.

Haemochromatosis and Haemosiderosis. — Haemochromatosis signifies a condition in which there is an increase in the total iron content of the body accompanied by cirrhosis of the liver. Although skin pigmentation, diabetes, and other endocrine manifestations are present in many cases they are not essential for a diagnosis of haemochromatosis. Haemosiderosis, on the other hand, merely implies an increase in the total iron content of the tissues without altered function or morphology (Aufderheide *et al.*, 1953).

Two types of haemochromatosis are recognized to-day: (1) a primary or endogenous type, and (2) a secondary or exogenous type. The aetiology of primary haemochromatosis is unknown, but anaemia is rarely present and there is a remarkable sex incidence of 20:1 in favour of the male sex. Secondary haemochromatosis has been found in association with prolonged anaemia, multiple blood transfusions, or both. Although these two types may be aetiologically dissimilar their clinical and pathological manifestations are the same, and, as reference to the table below indicates, there is no essential difference in the distribution of the haemosiderin and haemofuchsin in the body tissue.

It is interesting to note that some cases of exogenous haemochromatosis show a similar disorder of melanin metabolism as is seen in the primary form. Recently Nissim (1953) by parenteral administration of iron to animals has produced siderosis in sites which up to now have been exclusively confined to haemochromatosis. However, it has so far proved impossible experimentally to reproduce the disturbance of melanin metabolism seen in haemochromatosis of primary or secondary origin.

TABLE

COMPARISON OF PIGMENT DISTRIBUTION IN PRIMARY AND SECONDARY HAEMOCHROMATOSIS

	Primary Haemochromatosis	Secondary Haemochromatosis (Goldish and Aufderheide, 1953)	Our Case
Haemosiderin:			
Liver	+++	+++	+++
Pancreas	++	++	++
Spleen	++	++	++
Gastric mucosa	+ Usually	+	+
Salivary glands	+ ,,	+	+
Sweat glands	+ ,,	+	−
Myocardium	+	+	+
Voluntary muscle	+ Usually	Not stated	+
Pituitary	+ ,,	,, ,,	+
Thyroid	+ ,,	+	−
Adrenal	+ ,,	+	+
Testis	+ ,,	Not stated	(A female)
Kidney	−	+	•+++
Melanin:			
Epidermis	++	++	++

+++ = heavy deposition of the pigment. ++ = moderate deposition of the pigment. + = some excess of the pigment.

• The renal siderosis in our case is attributable to the paroxysmal nocturnal haemoglobinuria.

The total iron content of the body is about 5 g. of which about 2½ g. is in the form of circulating haemoglobin in the red cells. In our case the liver alone contained 21 g. In addition there was a well-marked portal cirrhosis giving abnormal liver function tests. Thus she had the two essential criteria necessary for a diagnosis of haemochromatosis.

In view of her anaemia and sex it appears most likely that this is a case of exogenous haemochromatosis, although the coincidental existence of a primary haemochromatosis and paroxysmal nocturnal haemoglobinuria cannot be absolutely excluded.

Iron Loss and Storage in Paroxysmal Nocturnal Haemoglobinuria.—Superficially it might appear that the continuous intravascular haemolysis in this disorder should lead to the presence of a haemosiderosis. This, however, is not the case. The pattern, in the absence of massive and multiple transfusion therapy, is one of renal haemosiderosis associated with depletion of the normal iron depots. This pattern is characteristic and specific and is in contrast to the other forms of chronic

haemolytic anaemia where the normal depots are full but the kidneys may contain little or no iron. The pattern of iron distribution seen is due to the almost constant haemosiderinuria which is characteristic of the disease. A daily loss of up to 5 mg. of iron may be incurred in this way, and when haemoglobinuria is present the total iron loss is much greater. Despite this loss patients with paroxysmal nocturnal haemoglobinuria are not generally iron deficient (Crosby, 1953), as there is a compensatory increase in iron absorption from the gastro-intestinal tract.

There are thus two points which require elucidation. The first concerns the aetiology of the haemosiderosis in a patient who was losing iron steadily through the kidneys for at least four years, and the second that of the portal cirrhosis.

Apart from primary haemochromatosis, to which reference has already been made, extensive haemosiderosis may be seen in patients who have received multiple and massive transfusion therapy (Schwartz and Blumenthal, 1948), or in patients suffering from malnutrition with pellagra (Gillman and Gillman, 1947).

That blood transfusion and "ferrivenin" therapy could not have contributed materially to the haemosiderosis is obvious from the fact that over the four years she was under treatment she received only 10 pints of blood, which would account for a mere 2.5 g. of iron and another 2.5 g. as "ferrivenin," whereas the liver alone contained 21 g.

Gillman and Gillman (1947) have described a condition of pigment cirrhosis in undernourished African natives. The liver pathology is indistinguishable from that seen in primary haemochromatosis. Our patient, however, showed no real evidence of general malnutrition, nor did her diet appear to have been inadequate or unbalanced.

Having excluded blood transfusion, "ferrivenin" therapy, and general malnutrition together or singly as sole factors in the aetiology of the haemochromatosis, we are forced to conclude that most of the iron must have been absorbed through the gastro-intestinal tract despite the fact that she did not require iron, as at no time was the anaemia a hypochromic one. Since normally, other than by haemorrhage, iron once absorbed cannot leave the body (Granick, 1954) the excess iron which was absorbed accumulated in the tissues, and caused a haemosiderosis and later a haemochromatosis.

Aufderheide *et al.* (1953) have reviewed the literature of exogenous haemochromatosis and have pointed out that the only common factor in all the cases described was the presence of a prolonged anaemia. They postulated that a prolonged anaemia causes a breakdown in the normal mechanism regulating iron absorption, leading to an excessive uptake of iron from the gastro-intestinal tract, even though the body does not require it. This iron accumulates and a haemosiderosis develops, and, if the patient lives long enough, portal cirrhosis and fibrosis in other organs may develop.

It is the degree and not the type of anaemia which is important. It may be macrocytic, microcytic, normocytic, aplastic, but, as long as the haemoglobin level remains low, iron will tend to be absorbed to excess. Studies with radioactive iron have shown that some anaemic patients continue to absorb appreciable quantities of iron though they do not require it (Dubach, Callender, and Moore, 1948). Experimentally the normal mechanism regulating iron absorption can be bypassed by massive doses of iron, low phosphorus intake, or both (Kinney, Hegsted, and Finch, 1949; Hegsted, Finch, and Kinney, 1949).

Our patient had a prolonged and severe anaemia, at times normocytic and at times macrocytic. She received large doses of oral iron (six to nine 3-gr. tablets of ferrous sulphate daily, almost continuously over a period of four years). Thus, the development of haemosiderosis in this case could be explained by the theory of excessive absorption of iron due to a chronic anaemic state, as in cases described by Aufderheide *et al.*

The aetiology of the portal cirrhosis is less obvious. The role of iron in the production of fibrosis is not as yet fully understood. Schwartz and Blumenthal (1948), with whom Aufderheide *et al.* agree, believe that the deposition of large amounts of iron in an organ or tissue will invariably induce fibrosis provided the patient lives long enough. Heitzman *et al.* (1953) and Norris and McEwen (1950) failed to find any constant relationship between the amount of iron deposited and the degree of fibrosis in an organ.

In the case under consideration it would appear that both the haemosiderosis and the portal cirrhosis may be explained on the basis of the prolonged and severe anaemia. The anaemia, by causing a local nutritional defect in the liver, may have initiated the portal cirrhosis, which in turn was accelerated and aggravated by the hepatic siderosis due to the increased absorption of iron from the gastro-intestinal tract. Thus a vicious cycle was set up which could only be broken by correcting the anaemia.

These observations are not merely of theoretical interest, since if correct they would suggest that the prolonged administration of iron to patients who are not responding to it may lead to haemochromatosis. Most chronic haemolytic anaemias do not show evidence of iron deficiency, so that rational treatment should exclude that with oral or intravenous iron. If there is evidence that the iron depots are full, as indicated by a raised serum iron level and demonstrable iron in a marrow section, a diet limiting iron absorption should be considered. This may be achieved by giving a diet low in iron and high in phosphates, especially phytic acid, which renders the iron insoluble (Granick, 1954).

Megaloblastic Anaemia in Paroxysmal Nocturnal Haemoglobinuria and Haemochromatosis.—A macrocytic anaemia may be encountered in liver disease; it is, however, generally macronormoblastic and very rarely megaloblastic (Movitt, 1950). Koszewski (1952) noted the presence of a megaloblastic anaemia in nine cases of haemochromatosis in a series of 35.

Two years after this patient came under observation she developed a megaloblastic anaemia. Free acid in the stomach and a 93% fat absorption excluded Addisonian anaemia and steatorrhoea respectively. The anaemia showed a partial response to vitamin B_{12} and liver extract, but complete remission only followed folic acid therapy. To what extent the paroxysmal nocturnal haemoglobinuria, by throwing an increased strain on the body's folic acid and vitamin B_{12} reserves, was responsible for the development of a megaloblastic anaemia we are unable to say.

Conclusion

The diagnosis of paroxysmal nocturnal haemoglobinuria was somewhat delayed in this case as initially the haemolytic features were not evident. The cirrhosis of the liver and later the megaloblastic anaemia appeared to provide a satisfactory explanation for the signs and symptoms. However, following the detection of haemoglobinuria the special tests for paroxysmal nocturnal haemoglobinuria were carried out and the diagnosis soon confirmed. The most valuable and specific test is Crosby's modification of Ham's test.

The post-mortem findings of intense renal siderosis without significant splenic enlargement and marked hyperplasia of the red bone marrow were consistent with this diagnosis, as in no other disease is this intense renal siderosis seen.

The concomitant finding of hepatic cirrhosis with marked haemosiderosis and siderosis of other organs, which constitute a haemochromatosis, was quite unexpected. The significance of the previously unexplained bronze pigmentation of the skin was now obvious. Haemochromatosis was not considered during life, as its development in a condition such as paroxysmal nocturnal haemoglobinuria, which is associated with a daily iron loss, would appear illogical.

It is clear from this case that a chronic anaemia is capable of upsetting the normal mechanism regulating iron absorption from the gastrointestinal tract; if the cells of the intestinal mucous membrane are then presented with large quantities of absorbable iron much of it will be taken up, even though it is not required. It seems that in the presence of a prolonged anaemia of any kind the body is incapable of discriminating between an iron-deficiency and a non-iron-deficiency anaemia.

Summary

A case of paroxysmal nocturnal haemoglobinuria is described in which a fatal anuria followed transfusion with washed blood cells.

At one stage the bone marrow showed megaloblastic erythropoiesis.

Necropsy revealed portal cirrhosis, intense siderosis of the liver and kidneys, and to a lesser extent of the spleen, pancreas, and other organs.

The mechanism for haemosiderosis is discussed in relation to current views on the regulating mechanism of absorption of iron from the gastrointestinal tract.

The part played by renal haemosiderosis in the production of kidney damage is discussed.

We wish to thank Dr. Oelbaum for permission to publish the case, Dr. J. Davson for his help and criticism, especially in the interpretation of the kidney lesions, Mr. H. V. Street for his help with the iron analysis of the liver, and Miss J. Perry, who took the photographs.

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THE VALUE OF A SERUM AND ALBUMIN MIXTURE FOR USE IN THE DETECTION OF BLOOD GROUP ANTIGEN-ANTIBODY REACTIONS

BY

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It has been suggested by Wiener and Hurst (1947) and Wiener, Hurst, and Sonn-Gordon (1947) that mixtures of bovine albumin and human plasma are more effective as diluents for the detection of incomplete antibodies than the widely employed 20% bovine albumin introduced by Diamond and Abelson (1945) or the human plasma alone used by Wiener (1945). This has been confirmed by other workers, and Mollison (1951) has recommended mixing a suspension of red cells in 20% albumin with the test serum diluted in group AB human plasma.

The present investigation was undertaken to determine whether the 20% bovine albumin test, which is open to criticism on a number of grounds, could be improved by substituting a mixture of human serum and bovine albumin for the diluent. Human plasma was not used because of clotting difficulties. After initial experiments it was decided to compare 20% bovine albumin with an equal parts mixture of human serum and 30% bovine albumin. Saline, and often human serum alone, was also included in the comparisons which were made with respect to different blood group antigen-antibody reactions.

Materials and Methods

Red Cells.—Red cells were obtained in the form of clotted blood. The cells recovered from the clot were washed three times in normal saline and packed. The supernatant saline was completely removed from the packed cells. They were then resuspended at 10% strength in the appropriate diluting fluid. The test cells were of group O unless otherwise stated.

Human Serum.—The serum used for diluting the bovine albumin was obtained from donors of group AB and was either fresh or had been kept frozen solid for varying periods of time, and is referred to as AB serum. It was free from all blood group antibodies and had no rouleau-forming properties.

Antisera.—These human sera contained Rh or other blood group antibodies of various strengths and had been preserved frozen solid.

Bovine Albumin.—Armour's 30% bovine albumin (or bovine plasma) was used in the tests. The 20% albumin was made by diluting this with half its

TABLE I

RESULTS OF VARIOUS TESTS PERFORMED ON SERA CONTAINING WEAK ANTIBODIES (ANTI-D)

Serum No.	Tests																
	Coombs Test			Papainized Red Cells		Agglutinins 37° C. in Saline			Titration of Serum in 20% Albumin × R_1r Cells at 37° C.					Titration of Serum in Serum-Albumin Mixture × R_1r Cells at 37° C.				
	R_1r	R_2r	rr	(R_1r+R_2r)*	rr	R_1r	R_2r	rr	1:1	1:2	1:4	1:8	Control	1:1	1:2	1:4	1:8	Control
16 A 14	++	++	–	++	–	–	–	–	–	–	–	–	–	+	–	–	–	–
16 A 43	+	+	–	++	–	–	–	–	–	–	–	–	–	+	w	–	–	–
20 F 15	++	+++	–	+++	–	–	w	–	w	–	–	–	–	+	–	–	–	–
20 F 12	–	+	–	+++	–	–	–	–	w	–	–	–	–	++	+	w	–	–
20 G 15	+++	+++	–	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20 G 44	++++	++++	–	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–

* Cell mixture.

volume of saline. The serum-albumin mixtures were made by mixing the necessary volume of serum with 30% albumin.

Technique.—The tests were performed in tubes 2 in. by ¼ in. One volume, 0.05 ml., of antiserum was placed in a tube and to this was added one volume of a 10% suspension of packed cells in the appropriate diluting fluid, i.e., 20% albumin, serum-albumin mixture, saline, or AB serum. (These tests correspond to the columns headed 1:1 in the tables of results.) Titrations were performed by making serial dilutions, viz., 1:2, 1:4, 1:8, and so on, of the antiserum in the appropriate fluid and adding one volume of cells suspended in the same fluid to one volume of each dilution. All these tubes were then tapped to mix the contents and incubated normally at 37° C., in previously warmed racks for two hours. Controls were put up with each titration consisting of one volume of diluent and one of cell suspension in the diluent. Control titrations were also performed using Rh-negative cells in place of Rh-positive ones where the test was concerned with an anti-Rh serum. Similarly, when other typing sera were being tested, positive and negative cells with respect to the particular antigen were used in parallel titrations as test and control. The results were read under the low-power objective of a microscope after gently spreading the sedimented clump from each tube on a slide with a pipette.

Results were scored as follows:

+ + + + + = Massive agglutination with no free cells.
+ + + + = Very strong agglutination with a few free cells.
+ + + = Many large clumps of cells clearly visible with more free cells.
+ + = Multiple clumps (10–12 cells) just visible to the naked eye, with many free cells.
+ = Small clumps (6–8 cells) visible microscopically; many free cells.
w = Clumps (3–4 cells) few in number; many free cells.
− = Negative; completely free cell suspension.

Anti-globulin or Coombs Test.—As described by Mollison, Mourant, and Race (1952), with modifications suggested by Stratton (1953).

Papainized Red Cells.—These were prepared and used as described by Stratton (1953).

Results

Preliminary Tests.—A large number of preliminary tests were made to find the best mixture of human serum and 30% albumin to use as a diluent in the subsequent comparisons with 20% albumin. The choice of an equal parts by volume mixture was made for the following reasons. It had the advantages of sensitivity and simplicity of preparation. Albumin-rich mixtures were difficult to work with and sedimentation of cells was slow in them owing to viscosity, while mixtures rich in serum lacked sensitivity. A series of experiments relevant to disadvantages in the 20% albumin test will now be detailed, in which this equal parts mixture of serum and albumin was compared with 20% albumin. The results are given largely in tabular form and discussed in the final section.

Detection of Weak Rh Antibodies.—Table I shows the results of testing sera containing weak anti-D antibodies by various methods. It will be observed that two sera negative in the 20% albumin test were weakly positive in the serum-albumin diluent, while two others giving very weak reactions in the former diluent gave stronger ones in the latter. All the antibodies in this table were weak but were detected using papainized cells and by the Coombs test. The last two sera in the table contained antibodies not demonstrated in the saline, 20% albumin or serum-albumin agglutination tests.

Enhancement of Titre of Rh-incomplete Antibodies by the Use of Serum-Albumin Mixture.—This enhancement of titre has been described before and was found to be usually two- to eight-fold. Fourteen incomplete anti-D sera of various titres were tested and all showed the greater effectiveness of serum-albumin in this respect. The control tests using Rh-negative cells all gave negative results. Table II illustrates a typical case.

TABLE II

SERUM NO. 20 F 3 (INCOMPLETE ANTI-D) TITRATED AGAINST Rh-POSITIVE R_1R_1 CELLS IN VARIOUS DILUENTS AT 37° C.*

Diluent	Dilutions								Control
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	
20% albumin	++++	+++	++	+	−	−	−	−	−
Serum-albumin	++++	++++	+++	+++	++	+	+	−	−
Saline	−	−	−	−	−	−	−	−	−
AB serum	+++	++	+	−	−	−	−	−	−

* Control titration against Rh-negative cells was negative throughout.

Table III

THREE SERA SHOWING ZONING IN 20% ALBUMIN TITRATED AGAINST VARIOUS CELLS IN DIFFERENT DILUENTS AT 37° C.

No.	Serum	Cells	Diluent	Dilutions																Control
				1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024	1:2,048	1:4,096	1:8,192	1:16,000	1:32,000	1:64,000	
a	No. 9 C 4 (Incomplete anti-C+D)	R_2r	20% albumin Serum-alb. Saline	++ +++ −	w ++ −	− ++ −	− +++ −	− +++ −	w +++ −	++ ++++ −	+++ ++++ −	+++ +++++ −	+++ +++++ −	++ ++++ −	+ +++ −	w +++ −	− ++ −	− ++ −	− + −	− − −	− − −
		R_1R_1	20% albumin Serum-alb. Saline	+++++ +++++ −	+++++ +++++ −	++++ +++++ −	+++ ++++ −	++ ++++ −	+ ++++ −	+++ ++++ −	+++ ++++ −	++ ++++ −	++ ++++ −	+ ++++ −	w +++ −	− +++ −	− ++ −	− + −	− − −	− − −
b	No. 10 F 60 (Incomplete anti-C+D)	R_2R_2	20% albumin Serum-alb. Saline	w +++ −	− +++ −	− ++++ −	− ++++ −	+ ++++ −	++ ++++ −	+++ ++++ −	+++ +++++ −	+++ +++++ −	++ ++++ −	+ ++++ −	w +++ −	− +++ −	− ++ −	− + −	− − −	− − −
c	No. 20 F 30 (Incomplete anti-D)	R_1R_1	20% albumin Serum-alb. Saline	w +++ −	++ ++++ −	+++ +++++ −	++++ +++++ −	+++ +++++ −	+++ ++++ −	++ +++ −	w ++ −	− + −	− w −	− − −	− − −	− − −	− − −	− − −	− − −	− − −

Table IV

TITRATIONS AT 37° C. PERFORMED TO INVESTIGATE THE BEHAVIOUR OF D^u CELLS IN VARIOUS DILUENTS

Serum	Cells	Diluent	Dilutions								Control
			1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
16 C 16 (Incomplete anti-D)	(a) $R_1{}^ur$ CD^ue/cde (Low grade)	20% albumin Serum-albumin Saline	− w −	− ++ −	− +++ −	− ++ −	− + −	− w −	− − −	− − −	− − −	− − −
	(b) Ordinary R_1r CDe/cde	20% albumin Serum-albumin Saline	++++ ++++ −	+++ ++++ −	+++ +++ −	+++ +++ −	++ +++ −	+ ++ −	− + −	− w −	− − −	− − −
	(c) $R_2{}^ur$ cD^uE/cde (High grade)	20% albumin Serum-albumin Saline	w ++ −	w +++ −	+ +++ −	+ ++ −	− ++ −	− + −	− − −	− − −	− − −	− − −
16 K 26 (Incomplete anti-D)	(d) $R_2{}^ur$ cD^uE/cde (High grade)	20% albumin Serum-albumin Saline	− + −	− + −	− + −	− − −	− − −	− − −	− − −	− − −	− − −	− − −

Occurrence of Zoning in Detection of Rh Antibodies.—Table III shows the titration of three anti-Rh sera with various Rh-positive cells in different diluents. All these sera showed zoning when the titration was performed using 20% albumin, but the zoning in each case was of a different kind and degree. In Table III (a), the zone using R_2r test cells was a marked one in the middle of the titration; it did not occur so markedly using type R_1R_1 (CDe/CDe) cells since the serum was of anti-C + D variety and zoning most commonly occurs only with respect to D. The serum in Table III (b) showed zoning starting earlier in the titration in 20% albumin. The third serum (Table III (c)) shows only a slight zone which is most marked in the lower dilutions, 1:1, 1:2, of the serum. It will be observed that the dilutions which gave negative results within the zones of the 20% albumin titrations gave unmistakable positive agglutination with serum-albumin. In the case of the second and third sera, if a single tube albumin test were used, the weak result at 1:1 might readily have passed unobserved, whereas with serum-albumin the corresponding result was +++. Serum 20 F 30 (Table III (c)), being anti-D unlike the other two sera, showed a zone in 20% albumin against both R_1 and R_2 type cells.

Detection of Reaction between Incomplete Rh Antibodies and D^u Cells.—It is known from previous work (Stratton and Renton, 1949) that D^u cells of low grade are not agglutinated by incomplete Rh antibodies in a 20% albumin diluent. Table IV (a) shows the titration of an incomplete anti-D serum using various diluents and cells of type R_1^ur (CD^ue/cde) low grade. Here the expected negative result was obtained using 20% albumin, but with serum-albumin the result was positive. It is interesting to note that this titration showed a zone. In Table IV (b) titration of the same serum with ordinary R_1r cells (CDe/cde) using the same diluents is shown. This serum was specially selected and known to be valuable in detecting D^u positive cells when using the indirect Coombs technique. When a number of other sera were used, some of lower titre, no agglutination was obtained even using serum-albumin diluent and CD^u/cde cells of low grade. Table IV (c) shows the results of titrating the same selected serum with cells of type R_2^ur (cD^uE/cde) high grade. Here an agglutination was obtained in both 20% albumin and serum-albumin diluents, but the titre with the latter is much higher and the individual results with each dilution stronger. When a different, incomplete anti-D serum of lower titre was titrated with these high grade D^u cells, a negative result was obtained using 20% bovine albumin, but a positive one using serum-albumin diluent (Table IV (d)).

Detection of Other Varieties of Rh Antibodies.—Other varieties of Rh antibodies were found to behave in a manner similar to the anti-D and anti-C + D antibodies which have been considered so far. An anti-c antibody (mainly incomplete) had a titre of 1 in 64 in 20% albumin, 1 in 256 in serum-albumin, and 1 in 4 in saline against Rh-negative (cde/cde) cells, and was completely negative with R_1R_1 (CDe/CDe) cells in these media. An anti-E antibody of titre 1 in 4 in albumin was of titre 1 in 16 in serum-albumin against R_2R_2 (cDE/cDE) cells and control titrations were again satisfactorily negative using R_1R_1 and Rh negative cells.

Detection of Complete Antibodies.—Two varieties of complete antibodies were examined; first, what was considered to be a serum containing largely complete Rh antibodies, in view of the fact that its titre was the same in saline, 20% albumin, and whole serum, namely, 1 in 32. The titre in serum-albumin mixture was 64.

Anti-A and anti-B antibodies, some of which had been selected as being of low titre in saline, were titrated out in various diluents against cells of appropriate group. There was no evident enhancement of agglutination using either 20% albumin or serum-albumin mixture and, indeed, with some sera the results were worse than when a saline diluent was used.

Anti-Kell.—Six anti-Kell sera were titrated with known Kell-positive cells in various diluents; the results of a typical titration are shown in Table V. It is interesting to observe the negative reaction in the 20% albumin and the positive result which was obtained in the serum-albumin titration; also an even stronger agglutination occurred in serum alone. Grove-Rasmussen (personal communication) informed us he had found this using several different anti-Kell sera.

TABLE V

TITRATION OF AN ANTI-KELL SERUM (NO. 13 F 1) WITH KELL-POSITIVE CELLS AT 37° C. IN VARIOUS DILUENTS

Diluent	Dilutions					Control
	1:1	1:2	1:4	1:8	1:16	
20% albumin ..	–	–	–	–	–	–
Serum-albumin ..	++	+	w	–	–	–
Saline	–	–	–	–	–	–
AB serum ..	+++	++	++	+	–	–

Five out of six anti-Kell sera we examined showed this higher titre in whole serum than in any other diluent.

Anti-Duffy, Anti-S, and Anti-Kidd.—Several examples of each of these sera were tested with the various diluents, but all gave negative results with cells containing the agglutinogen for these antisera.

Anti-Lewis.—Various tests have been made titrating anti-Le^a and anti-Le^b antibodies with the appropriate cells at 16° C. and 37° C., using the usual diluents. The results showed little enhancement of agglutination when 20% albumin or serum-albumin was used, compared with saline, but it is probably advantageous to use an albumin technique at 37° C. if it is suspected that incomplete Lewis antibodies are present in a serum.

Effect of Albumin Mixture on Activity of Cold Agglutinins.—Table VI shows the titration of a non-specific cold antibody at 8° C. and 37° C. There is little difference between the titres and strengths of agglutination using 20% albumin and serum-albumin, but both give a higher titre than saline.

TABLE VI

TITRATION OF SERUM (NO. 12 D 17) CONTAINING A NON-SPECIFIC COLD AGGLUTININ WITH Rh-NEGATIVE CELLS AT 8° C. AND 37° C. IN VARIOUS DILUENTS

Diluent	Dilutions						Control
	1:1	1:2	1:4	1:8	1:16	1:32	
(a) 8° C.:							
20% albumin	++++	+++	+++	++	w	–	–
Serum-albumin	++++	+++	+++	++	+	–	–
Saline ..	+++	++	+	+	–	–	–
(b) 37° C.:							
20% albumin	–	–	–	–	–	–	–
Serum-albumin	–	–	–	–	–	–	–
Saline ..	–	–	–	–	–	–	–

Centrifugation Experiments.—Experiments were carried out to see whether time could be saved during the performance of albumin tests by centrifuging the tubes following a limited incubation period and then reading the tests. An anti-D serum titrated in both 20% albumin and serum-albumin mixture was employed. The tubes were incubated for 20 minutes and then centrifuged at low speed (500 r.p.m.) for five minutes and the results read. Comparison with the standard method showed that no serious error would result from using this method if a result is required quickly. Centrifugation experiments have been conducted with only a limited number of anti-Rh sera and not with the full series of tests.

Commentary

The albumin test, which is widely used for routine purposes, suffers from certain defects. Some of these can be remedied by using serum-albumin mixture in the proportions suggested instead of the 20% albumin mixture normally used.

It has been shown (Stratton, 1953) that as many as 5 to 7% of weak Rh antibodies in the maternal serum might be missed if the 20% bovine albumin test were used alone ; when a serum-albumin mixture is used many fewer Rh antibodies would remain undetected.

All serological techniques for the detection of blood group antigen-antibody reactions are liable to give false negative results due to the occurrence of zoning, but the albumin test is particularly prone to this phenomenon. This means that when using a 20% bovine albumin diluent a single tube test cannot safely be employed and the test should be performed in the form of a titration using up to six tubes. The results reported here show that a serum-albumin diluent reduces the danger of these false negative results. Even where the first tube of a serum titration shows a weak positive result, due to zoning when 20% bovine-albumin is used, the substitution of the serum-albumin mixture would result in a stronger positive result. It should not be assumed, however, that if a serum-albumin mixture is used every difficulty due to zoning will be eliminated in this test, but false negative results due to this cause will be considerably diminished.

Stratton and Renton (1949) showed that the D^u type of cells, especially if of low grade, will not react with incomplete anti-D sera in 20% bovine albumin, but if the D^u type of cell is of high grade occasional positive reactions will be obtained depending upon the incomplete anti-D serum that is used. When a serum-albumin diluent is used, depending upon the particular anti-D sera, some low grade D^u positive cells may be detected ; even so, a zone may occur in these titrations. Nevertheless, it would seem that, in a population in which there occurs a large percentage of the D^u-positive type of person and where the albumin test is used as a screening test in the first instance for the detection of D-positive persons, materially better results would be obtained if a serum-albumin mixture were used for the original screening test. It should be emphasized that negative results would need to be retested using the Coombs test. One should not attempt to detect low-grade D^u cells using the albumin technique alone, whatever diluent is used.

Rarely, Rh antibodies are not detectable by the antiglobulin or other tests, but only using the albumin technique. If these Rh antibodies are detected in the serum of an Rh-negative mother during the antenatal period, the subsequent sample usually gives a positive indirect Coombs test. Anti-E antibody is one that is most often preferentially detected by the use of the albumin technique.

Kell is an antigen to which persons readily become immunized and anti-Kell antibodies are often detected in human sera. The Coombs test has most often been used for the detection of these antibodies, and the 20% bovine albumin test does not usually give a positive result. Our results suggest, however, that if serum-albumin is used positive results will be obtained. Grove-Rasmussen (personal communication) suggested that good results were obtained if a serum diluent were used, and this has been confirmed.

Anti-Duffy, anti-Kidd, and sometimes anti-S cannot be detected at all using the albumin technique, whichever diluent is used, and the chances of detecting Lewis antibodies are not materially increased by the substitution of a serum-albumin mixture for a 20% one.

In the case of anti-A and anti-B saline agglutinins, our impression, after testing many samples, was that these agglutinins are not as readily detected in albumin or in serum-albumin mixtures as they are in saline, although they would be unlikely to give completely negative results in the first two diluents and positive ones in saline.

Experiments were carried out to determine whether the increased rate of sedimentation of the red cells was the sole reason for the enhanced agglutination of the serum-albumin mixture as compared with 20% albumin. These experiments consisted of reading results of parallel titrations after incubation periods ranging from one to four hours. There was no evidence to support this, since in all cases the serum-albumin results were stronger than those where 20% albumin was used. These experiments did suggest that economies in time, such as reading the test after one and a half hours' incubation, would not have such serious effects in preventing the detection of positive results if serum-albumin were used as a diluent compared with 20% bovine albumin. It seems to us, therefore, that the time of incubation might be reduced accordingly when serum-albumin mixture is used. Low-speed centrifugation to reduce the time of incubation was also considered and an incubation time of 20 minutes at 37° C. in the water-bath followed by low-speed centrifugation (500 r.p.m.) might well be used where speed is desired. Our tests with this technique have only been concerned with a limited number of Rh antibodies.

Many observers have shown that other substances will act in the same way as bovine albumin and human plasma or serum. Gelatin and dextran, for example, have been employed, and one of us found, of many substances tried, that methyl cellulose was the best. All these substances suffered from the defect that they tend to cause false positive results which make the interpretation of the test most difficult. The selection of a suitable test medium, therefore, involves finding one which will not give false positive results but will detect blood group antigen-antibody reactions with the minimum of difficulty.

In the authors' experience it is an advantage to add human serum in the proportions suggested to bovine albumin, and such a 50/50 mixture will not increase the false positive results or the difficulty of reading the test. The serum used in making the mixture should be free from blood-group antibodies and all rouleau-forming properties, and we are in favour of freezing it solid at least a week or ten days before making up the mixture. After mixing it is stored at 4° C. and not kept for more than a few days.

The reading and interpretation of the albumin test have always presented difficulty. The cell deposit should be carefully removed from the tube and spread upon a microscope slide. At this stage aggregation of the red cells is liable to be present, especially after centrifugation, and gives the effect of comets, i.e., a clump of cells from which a tail of cells can be seen trailing away. It is important to leave the slide a few minutes on the bench before placing it under the low power of the microscope and then to examine a moving film. This is done by tilting the slide on the stage when the break-up of the cell aggregates will be observed, the true agglutinates remaining intact. In the case of saline agglutination tests, it is often possible to distinguish rouleau from true agglutination microscopically, but in the case of albumin or serum-albumin tests it is quite impossible to make this distinction.

The albumin technique is widely employed in the crossmatching test and often in the form of a single tube test. While the authors would not

recommend this as a routine method of cross-matching, nevertheless it does seem a considerable advantage to use the serum-albumin mixture suggested in place of 20% bovine albumin in such a test, and such a mixture would also be advantageous if the test were done in the form of a titration. In the crossmatching test, however, it seems preferable to use a saline agglutination method in tubes at room temperature and at 37° C. coupled with the use of the antiglobulin technique.

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THE CORRECTION OF MEAN CELL VOLUME AND MEAN CELL HAEMOGLOBIN CONCENTRATION

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The determination of red cell constants is important in the diagnosis of certain blood disorders and in following the response to therapy. The accuracy of the mean cell volume (M.C.V.) and the mean cell haemoglobin concentration (M.C.H.C.) is dependent on the precision and reproducibility of the individual estimations. It is widely accepted (Wintrobe, 1951 ; Whitby and Britton, 1953) that the haematocrit determination (<1% error) is more accurate than the haemoglobin estimation (2–3% error) and considerably more accurate than the red cell count (7–10% minimum error). In this respect, we may confirm the reproducibility of the haematocrit determination since duplicates, set up in 3 mm. Wintrobe tubes and spun simultaneously in the same centrifuge, agree within less than 0.5 divisions, read to the nearest tenth of a division by microscopic magnification against an eyepiece scale.

However, while techniques for haemoglobin estimation and red cell counting are standardized, those for the haematocrit determination are less so. Haematological and clinical pathological texts, among others those of Dacie (1950), Wintrobe (1951), and Whitby and Britton (1953), quote a variety of speeds and times of centrifugation, usually 20–30 minutes at 2,000–3,000 r.p.m. Nevertheless, the actual completeness of packing depends on the size and shape of the red cells and the centrifugal force applied and its duration. Oxygenated red cells are smaller than cells containing a high concentration of reduced haemoglobin, while only heparin and the ammonium-potassium oxalate mixture as anticoagulants do not alter red cell volume (Adams and Mosher, 1951 ; Bernstein, 1953).

Experimental

Fresh aerated heparinized blood samples from male medical students were divided and spun in capped 3 mm. Wintrobe tubes in a standard laboratory centrifuge with a 15 cm. radial head. The tachometer speeds were checked stroboscopically. The packed cell column decreased rapidly in tubes spun for 45 min. between 2,000 and 3,000 r.p.m. (relative centrifugal force to bottom of tube, 670–1,510 g), with a lesser decrease between 3,000 and 4,500 r.p.m. (1,510–3,400 g). Representative results are detailed in Table I, together with the mean relative haematocrit values for 22 samples, taking the haematocrit reading at 4,500 r.p.m. as 100.

TABLE I

HAEMATOCRIT OF NORMAL BLOOD SAMPLES SPUN AT VARIOUS CENTRIFUGAL FORCES

Speed of Centrifugation (r.p.m.)	Relative Centrifugal Force* (g.)	Haematocrit Sample 1 (%)	Haematocrit Sample 2 (%)	Mean Relative Haematocrit†
1,500	378	54·8	57·6	122
2,000	670	51·6	55·0	116
2,500	1,050	48·8	52·5	109
3,000	1,510	47·4	49·8	105
3,500	2,055	45·6	48·2	102
4,000	2,684	45·0	47·6	101
4,500	3,400	45·0	47·4	100

* Calculated for radial distance (15 cm.) to bottom of Wintrobe tube.

† Haematocrit at 4,500 r.p.m. taken as 100.

The duration of spinning at any centrifugal speed also influences the packed cell column ; there is a moderate decrease between 20 and 30 minutes, with little further change between 45 and 90 minutes (Fig. 1).

The degree of red cell packing was ascertained by direct estimation of the trapped intercellular plasma, using inulin or T1824 as plasma-marking agents. For example, 1/10 volume of 0.5% Evans blue (T1824) dye was mixed with the heparinized specimens and placed in 3 mm. Wintrobe tubes in duplicate for centrifugation at different speeds and times. Supernatant plasma (0.1 ml.) was serially diluted 1/250, 1/500, 1/1,000 in 0.9% NaCl to provide individual standards for reading each test sample (Evelyn photometer, 620 mμ, 6 ml. aperture, 2 cm. cells).

The dyed plasma remaining in the haematocrit was discarded by adding saline with careful successive aspiration, followed by removal of the buffy coat and superficial red cell layer. The red cell level was noted for calculation of cell volume (v_{RB}-)—for example, $3.1416 \times (0.15)^2 \times 4.3 = 0.304$ ml. The haematocrits

were weighed with mercury to the 4 and 5 cm. marks to check the volume.

The packed cells were carefully removed to a 10 ml. volumetric flask, the haematocrit and aspirating pipette being repeatedly rinsed with saline, and the density of the centrifuged supernate read. The trapped intercellular plasma in ml. is calculated:

$$0.1 \times \frac{T}{S} \times \frac{10 - V_{RBC}}{V}$$

where T and S = density of test and standard respectively

V = volume of diluted standard, i.e. 25, 50, or 100

and is expressed as a percentage of the volume of red cells (V_{RBC}).

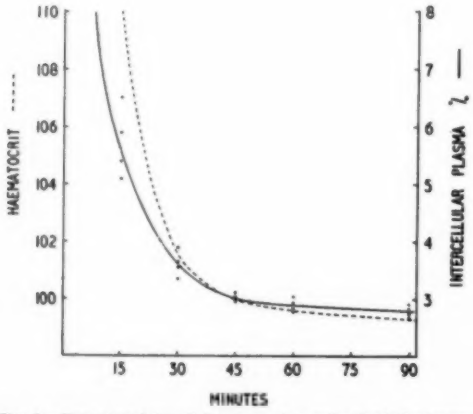

FIG. 1.—Percentage intercellular plasma trapped and relative haematocrit values for four normal blood samples, centrifuged for different times at 3,500 r.p.m., radial head 15 cm. (×2,050 g).

The plasma volume trapped in the red cell mass was 4–12% at 2,000 to 3,000 r.p.m. spun for 45 to 60 minutes with a 10 to 20% coefficient of variation, in contrast to the 1.8–3% trapping at the higher speeds with a 5% coefficient of variation. Thus the amount of plasma trapped intercellularly, and, more particularly, the degree of variation in packing, was of a very variable order with the slower rates of spinning. Recent reviews and investigations (Gregersen, 1951; Leeson and Reeve, 1951; Chaplin and Mollison, 1952) indicate that a force of at least 2,000 g is required to obtain reproducible packing of red cells. Our results confirm such a conclusion, and centrifugation at 3,500 r.p.m. with a 15 cm. head (×2,050 g) for 45 minutes has been adopted as routine procedure.

For bloods with widely differing packed cell columns spun under standard conditions the observed trapped intercellular plasma decreases with reduction in the haematocrit reading. This ensues from the increase in centrifugal force (calculated to the mid-point of the cell column) being applied to decreased packed cell columns (Table II).

TABLE II

INFLUENCE OF VARIATION IN HAEMATOCRIT AND RESULTING CENTRIFUGAL FORCE ON TRAPPED INTERCELLULAR PLASMA

Observed haematocrit (%) ..	65	50	35
Mid-radius of centrifugation (cm.)	11·75	12·50	13·25
Relative centrifugal force (g) ..	1,610	1,710	1,815
Observed trapped intercellular plasma (%)	3·5	2·8	2·2
Corrected haematocrit (%) ..	62·7	48·6	34·2

Comment

The mean haematocrit for the 22 samples was 50.1, range 45.2–53.8, S.D. 2.1 (altitude 5,740 ft.) and the corrected haematocrit 48.5. The corrected M.C.V. would be some 4% lower and the M.C.H.C. some 3% higher for these normal specimens. The correction would be greater for polycythaemic blood and less in anaemias. Provided that the haematocrit is centrifuged for at least 45 minutes at 3,500 r.p.m. in a 15 cm. radial head, the error in neglecting the trapped intercellular plasma in reading the packed cell column is relatively constant, so that reports from different laboratories are comparable. Under other conditions of centrifugation, the errors are variable and considerable.

Apart from the necessity for standardizing the conditions of the haematocrit determination in the calculation of haematological indices, the correction of the packed cell volume for the amount of trapped intercellular plasma is of particular importance in estimating (*a*) the total blood volume after cell or plasma volume has been determined, and (*b*) the distribution of solutes between blood cells and plasma (Bernstein, 1952).

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THE NORMAL BLOOD CLOTTING TIME IN THE LIGHT OF EXPERIENCE WITH THE "TWO-SYRINGE" TECHNIQUE

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The surgeon who requests the pathological laboratory to determine a patient's blood clotting time expects a result which will indicate if surgery might be dangerous. If he follows the teaching of Cummine and Lyons (1948) or of Dutton (1954) he may wish to use the test to anticipate thromboembolic episodes. The physician may request the test for various reasons. It is, accordingly, very important to know the upper limit of normal, which is stated by Wintrobe (1951) to be 15 minutes using the method of Lee and White (1913). Other textbooks give lower figures. Friedman, Schwartz, and Vincenti (1952), using a two-tube Lee-and-White technique, made the range of normal 7 to 25 minutes. They regarded the test as affording "little if any useful function in evaluating the clotting mechanism, especially in relation to the bleeding and clotting tendencies."

The work of Allen and Attyah (1953) suggested a possible explanation of these inconsistencies. These workers claimed that, even when a vein was entered with minimal trauma, blood withdrawn into the first syringe clotted sooner than that withdrawn subsequently into a second syringe. Normal medical students were used in their investigations. With large-calibre short tubes (internal diameter 9.5 mm., height 40 mm.) and 10 ml. syringes with 20-gauge needles, they showed that the coagulation time of the blood in the second syringe was significantly longer than that in the first. Each of 10 tubes was filled from the bottom with 1 ml. using an 18-gauge needle, and the whole test was performed in a water-bath at 37° C. The end-point was taken at the time for the blood in the last tube to clot, each being completely inverted at intervals of five minutes. The tubes were examined in the order in which they had been filled. In 96% the clotting time was 20–35 minutes; it was never under 15 or over 40 and the average was 26 minutes.

Method of Investigation

The blood coagulation times of new patients admitted to a large mental hospital were estimated. None of those investigated was physically ill and none gave a history of bleeding after operation. Advantage was taken of the fact that all patients admitted have blood taken for Wassermann and Widal tests, and the coagulation times were determined with these specimens of blood. The patients were not, therefore, inconvenienced. Those with difficult veins were excluded.

Test-tubes (not moistened with saline) measuring 3 in. by ¼ in. were filled from the bottom using 10-ml. syringes and 18-gauge needles. Blood was aspirated, with minimal venous trauma, into a syringe, which was then removed and a second one fitted on to the needle, which was maintained as motionless as possible in the vein. Clean needles were placed on each syringe and 1 ml. of blood was put into each tube; in all cases blood from the second syringe was put into 10 tubes, but blood from the first was usually only put into five. When blood appeared in each syringe a stop-watch was started. The tubes were left undisturbed for five minutes and then examined by tilting at intervals of 30 seconds. They were examined in the order in which they had been filled, each succeeding one being left undisturbed until blood in the preceding one had clotted. In taking the specimens suction was never applied until the tip of the needle was inside the lumen of the vein.

Results

As there is a difference of opinion as to how the coagulation time should be read, results are given both as the average of the tubes and also as that of the last tube.

Early in the investigation it became apparent that even slight movement of the needle in the vein made the clotting time of blood in the second syringe more or less the same as that in the first, whereas the less movement permitted the greater was the difference between the two samples. This

difference is shown in Table I, which also shows that, depending upon the way in which the results were expressed, the longest times were 18–21 minutes.

TABLE I
COAGULATION TIMES (IN MINUTES) OF BLOOD IN FIRST AND SECOND SYRINGES

No Perceptible Needle Movement				Slight Needle Movement			
1st Syringe		2nd Syringe		1st Syringe		2nd Syringe	
Average	Last Time	Average	Last Time	Average	Last Time	Average	Last Time
9	10	13	14	8	8½	8	8
10	10	15	17	5	5	5	5
10	10	15	15½	12½	14½	10	10
6	6	13	14	8½	8½	9	10
13	13	19	21	13	13	9½	12
10	10½	17½	19	8½	9	8	8
12½	13½	16	18	7	8	7½	7½
13	14	18	21	9	10	10	11
6	6	11	13	13	15	10	10
9½	10	13	15	15½	17	15½	17
7	7	11	12	9½	9½	9½	9½
7	8	13	14	10½	12	13	13
12½	14	17½	20½				
6½	6½	11½	12				
9½	9½	13	13				
5	5	10	12				
5	5	11	11				
5	5	16	17				

It should be made clear, however, that the 3 in. × ½ in. tubes which were used will have given different values from the standard 2½ in. × ¾ in. tubes.

Discussion

The work of Allen and Attyah (1953) has been confirmed in so far as the clotting time of blood in the second syringe has been found to be longer than that in the first. It was difficult to keep the needle absolutely motionless in the vein, and I find it hard to believe that this ideal was realized by Allen and Attyah in every case.

Wright, Maizels, and Jepson (1952) state that "the dominant factor normally controlling the clotting time of whole blood is the concentration of thromboplastin." It is reasonable to infer that, in those cases in which the needle was moved in the vein while changing syringes, thromboplastin was liberated into the lumen thereby shortening the clotting time. Prothrombin consumption tests were not performed in the course of the present investigation. However, it is most unlikely that the patients with clotting times exceeding 15 minutes were all suffering from asymptomatic haemorrhagic diatheses with prolonged clotting times. The "two-syringe" method shows the great significance of venous trauma upon the result. It seems very doubtful if methods of determining the clotting time using capillary blood are of any use, as contamination with thromboplastin is so likely to occur. In a patient with veins that are difficult to find, the intravenous technique will give results which are almost certainly too low. It is suggested that pathologists should always state in their reports whether the vein was entered with ease or not; the clinician will then be able to evaluate the result more accurately.

Summary

Estimations of the coagulation time, using a "two-syringe" modification of Lee and White's method, were performed on 30 physically normal patients newly admitted to a mental hospital. In 18 cases in which the technique was reasonably perfect, evidence was obtained that the coagulation time of blood in the second syringe was longer than that in the first. The results were the same in the 12 cases in which the needle was inadvertently moved slightly when the syringes were being changed. It is concluded that even slight injury to the intima of a vein liberates sufficient thromboplastin to shorten the coagulation time significantly. Evidence is produced to show that the upper limit of the coagulation time of normal venous whole blood is longer than that quoted in textbooks if care is taken to minimize the liberation of thromboplastin from the intima of the vein.

I wish to thank Dr. H. B. Miller for his encouragement to carry out research on his cases, and Dr. A. J. Shillitoe for his constructive criticism.

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COMPARISON OF SOLID AND LIQUID MEDIUM SENSITIVITY TESTS OF TUBERCLE BACILLI TO *PARA*-AMINOSALICYLIC ACID

BY

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In the treatment of tuberculosis *para*-aminosalicylic acid (P.A.S.) is now chiefly used in combination with streptomycin or isoniazid with the aim of preventing or delaying the emergence of drug-resistant strains of tubercle bacilli (Medical Research Council, 1950, 1953c). Such combined therapy is only effective in preventing drug resistance when the strains obtained before the start of treatment are sensitive to P.A.S. Thus resistance to streptomycin has been found to emerge more frequently during treatment with the combination of streptomycin and P.A.S. when the pre-treatment strains were resistant to P.A.S. than when they were sensitive to it (Medical Research Council, 1953b).

The selection of the most suitable chemotherapy for any patient should involve a test of the P.A.S. sensitivity of pre-treatment strains, since resistance would prejudice the results of treatment with a combination of drugs that included P.A.S. Initial P.A.S. resistance occurs most frequently as a result of previous treatment with P.A.S. alone. Less frequently it is found as a result of therapy with a combination of two drugs, one of which was P.A.S. Djang, Bogen, and Will (1952) have found some bovine strains to be naturally resistant to P.A.S. Finally, the patient may have been infected by another person who harboured a P.A.S.-resistant strain (Medical Research Council, 1953a ; Thomas, Borthwick, Horne, and Crofton, 1954). Routine P.A.S. sensitivity tests during chemotherapy are less valuable, but, if the patient has remained culture positive, a test at the end of a course of treatment may be very helpful in planning further treatment. Thus cultures from the majority of patients receiving chemotherapy should be tested on at least one occasion for P.A.S. sensitivity. The number of these tests may be a considerable burden for the pathologist, who will, therefore, look for a simple yet reliable technique of performing them.

During the Medical Research Council (1950, 1952b, 1953b) trials of streptomycin and isoniazid, P.A.S.-sensitivity tests were carried out in Tween-albumin medium. This method was convenient since many of the strains were also tested for streptomycin sensitivity in the same medium. On the other hand, American and French workers (American Trudeau Society, 1952 ; Coletsos, 1952) have advised the use of solid medium tests, claiming that these were more reliable and easier to use. A comparison of the two methods was clearly required both to establish their inherent accuracies and to provide the data necessary for recommending a simple technique that could be relied upon in testing an increasing number of cultures. Such a comparison has therefore been made, using, in the main, strains from patients treated in the Medical Research Council (1952b) trials of isoniazid.

Methods

Sensitivity Tests.—Liquid medium sensitivity tests to P.A.S. were carried out in Tween-albumin liquid medium by the Medical Research Council (1953a) method. In the solid medium tests, sodium *para*-aminosalicylate dihydrate (Na P.A.S.) was added to Löwenstein-Jensen medium before inspissation to make a series of final concentrations from 0.12 μg./ml., increasing by serial twofold steps, to 128 μg./ml. This medium was distributed in 4 ml. amounts in screw-capped bottles and inspissated at 80° C. for one hour. The inoculum was prepared by grinding up a representative loopful of the growth from a two to three week Löwenstein-Jensen slope culture in sterile distilled water to make a suspension approximately equal in opacity to a 10-day growth in Tween-albumin medium. A 3 mm. loopful (0.005 ml.) of this suspension was spread on each of the series of slopes containing P.A.S. and on a drug-free slope. Each batch

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of tests included one on the standard sensitive strain, H37Rv. Results were read after 14, 21, 28, and 42 days' incubation.

Cultures.—A total of 130 strains of tubercle bacilli was obtained from the sputa of patients with pulmonary tuberculosis. These were divided into three groups :—

Group I.—Forty-five pre-treatment strains from 45 patients who had received no previous chemotherapy.

Group II.—Thirty-six strains from 29 patients which were resistant to P.A.S. (resistance ratio of 8 or more) when tested in liquid medium.

Group III.—Forty-nine strains from 37 patients which were sensitive to P.A.S. in liquid medium, but, for reasons indicated in Table I, might have been resistant if tested by a more delicate technique.

TABLE I

REASONS FOR SUSPECTING P.A.S. RESISTANCE IN GROUP III STRAINS

	No. of Strains
P.A.S. resistance ratio of 2 or 4 in liquid medium test	8
P.A.S. resistance found by other laboratories	5
Previous culture from same patient found resistant to P.A.S.	6
Later culture from same patient found resistant to P.A.S.	3
Resistant to streptomycin	6
Previous chemotherapy with P.A.S. and isoniazid for 4–5 months	10
Previous chemotherapy with P.A.S. and isoniazid for 6 or more months	11
Total	49

The sensitivity of the majority of the strains was tested once in liquid medium. Each strain was then subcultivated on a Löwenstein-Jensen slope, and from the growth a further liquid medium test and a solid medium test were set up in parallel. The results of these parallel tests were compared.

Tests on 23 out of the 130 strains were carried out only once in liquid medium, and the strains were then subcultivated and tested on solid medium. The results of these tests were also included in the comparison. It might be thought that the inclusion of these results would bias a comparison between the methods, since it will be shown that some resistant strains tend to revert to sensitivity during subcultivation. However, no evidence of reversion was found among these strains; five of them were highly resistant in both tests and a sixth was more resistant in the second test than in the first. The remainder consisted of 11 pre-treatment strains which were unlikely to alter in sensitivity during subcultivation and six strains which were unequivocally sensitive in both tests.

End-points.—The minimal concentration of P.A.S. inhibiting the growth of resistant strains was frequently difficult to define, both in liquid and on solid media, since increasing P.A.S. concentrations only gradually decreased the rate of growth. In liquid medium tests it was important to read the presence or absence of growth under constant lighting conditions. The minimal concentration completely inhibiting growth was taken as the end-point. In solid medium tests two end-points were used. One of these was the minimal concentration necessary to inhibit growth completely. The other was the minimal concentration that prevented the growth of 20 or more colonies (subsequently called the "20 colonies end-point"). Scanty, minute colonies often appeared first on slopes containing P.A.S., and were then followed by a profuse growth over the whole inoculated surface. Since it was difficult to be certain that the minute colonies were composed of tubercle bacilli, the "20 colonies end-point" was found easier to use.

Results

Pre-treatment Strains.—Fig. 1 shows the results of sensitivity tests on pre-treatment strains (Group I) in terms of the minimal concentration of P.A.S. inhibiting growth. The shape of these distributions approximates to that of the normal curve of errors. The scatters of the distributions for tests in liquid or on solid medium were very similar. However, inhibitory concentrations were slightly lower in liquid medium. Thus, after 14 days' incubation in liquid medium, all the strains were inhibited by 4 μg./ml. Na P.A.S., whereas, after 28 days' incubation on solid medium, they were all inhibited by 16 μg./ml. Na P.A.S.

During incubation on solid medium growth appeared in progressively higher concentrations of P.A.S. Thus, between readings at 14 and 42 days, the minimal inhibitory concentrations increased on the average about four times. This was not due to inactivation during incubation, since the H37Rv strain was inhibited by the same P.A.S. concentration when the slopes were incubated for two, four, six, or eight weeks before inoculation. Singh (1955) has also shown that P.A.S. was not inactivated during incubation in liquid medium. Examination of the 28-day readings on solid medium showed that the "20 colonies end-point" was, on the average, one concentration lower than the "no-growth end-point."

Fig. 2 illustrates the results of sensitivity tests on the same strains, in terms of the resistance ratio, which is the ratio between the minimal inhibitory concentrations of the test strain and of the standard sensitive strain, H37Rv. Again, the shape and scatter of the distributions are very similar. Both in liquid and on solid medium all the strains had a resistance ratio of less than 8. Resistance ratios of $\frac{1}{2}$ were more common than those of 2, due to slightly lower minimal inhibitory concentrations for test strains than for H37Rv. It was presumed that test strains were less well adapted to growth

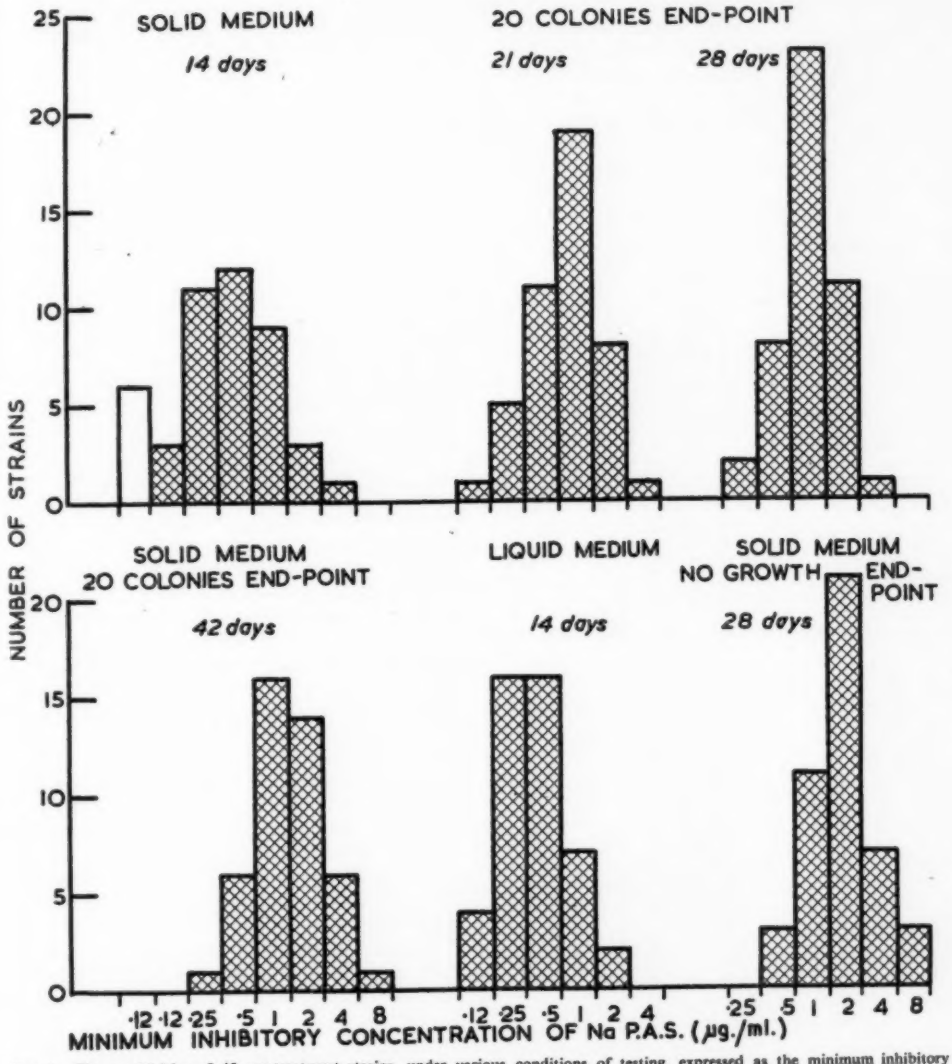

FIG. 1.—The sensitivities of 45 pre-treatment strains, under various conditions of testing, expressed as the minimum inhibitory concentrations of Na P.A.S. The result on one strain was not read after 42 days' incubation.

on artificial media than was H37Rv. The distributions of resistance ratios determined on solid medium after 21 or 42 days' incubation were similar to those at 28 days, but in about a third of the tests read after 14 days a resistance ratio could not be defined because of inadequate growth either of the test strain or of H37Rv.

Differences in Sensitivity of Pre-treatment Strains.—Sensitivity tests in liquid medium were carried out twice on 24 of the 46 pre-treatment strains. This made it possible to determine whether the apparent differences of sensitivity of these strains were due to the technical error of the tests or to real differences in the sensitivities of the

strains. Resistance ratios of $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$ . . . were scored as 1, 2, 3 . . . , and an analysis of variance was then carried out on these scores. The results are shown in Table II. The mean square for variation between strains was not significantly different from the error mean square, which is a measure of the technical error. One can therefore conclude that, as far as can be determined by the method

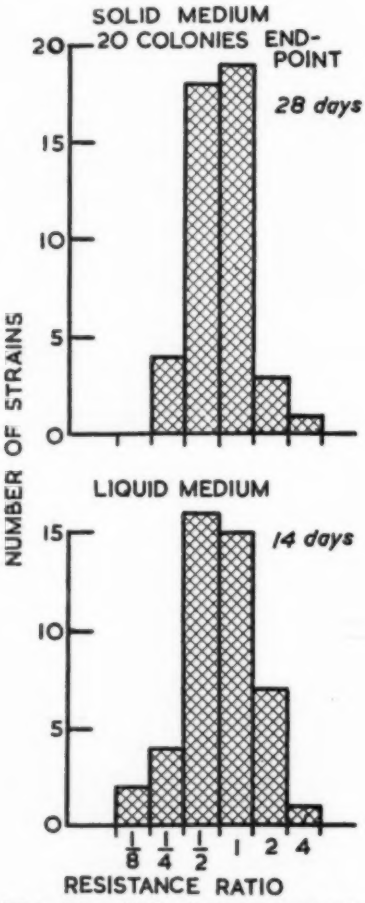

FIG. 2.—The sensitivities of 45 pre-treatment strains on solid and in liquid medium tests expressed as resistance ratios.

used, pre-treatment strains had the same degree of sensitivity to P.A.S., any apparent differences being due to technical error.

The standard deviation of the test is seen to be ±1.083, so that approximately 19 out of 20 tests

TABLE II

ANALYSIS OF VARIANCE OF DUPLICATE TESTS ON 24 PRE-TREATMENT STRAINS IN LIQUID MEDIUM

	Sum of Squares	Degrees of Freedom	Mean Square
Between repeat tests . .	2·083	1	2·083
,, strains	35·667	23	1·551
Error	25·917	23	1·083

lay within the range of four times more to four times less than the true value of their resistance ratio. An estimate, by similar means, of the standard deviation in streptomycin sensitivity tests carried out in Tween-albumin medium yielded a value of ±0.636 (Mitchison, 1949) so that the P.A.S. sensitivity test had about half the accuracy of the corresponding streptomycin sensitivity test.

Medium Batch Differences.—During the period of the experiments pre-treatment strains were tested on nine different batches of solid medium and in 12 different batches of liquid medium, all prepared in the same laboratory. Since pre-treatment strains had the same degree of sensitivity, an analysis could be made to determine whether the minimal concentration of P.A.S. that inhibited growth varied from batch to batch of medium. The results of solid medium tests read after 28 days, using the "20 colonies end-point," and of liquid medium tests read after 14 days were given scores as before. Minimal inhibitory concentrations of 0.12, 0.25, 0.5 . . . μg./ml. Na P.A.S. were scored as 0, 1, 2, . . . Analyses of variance were carried out, with the results shown in Table III. The mean square for variation between batches was significantly greater than the error mean square in the solid medium tests, but not in the liquid medium tests. One can conclude that for the purpose of these tests Löwenstein-Jensen medium was more difficult to standardize than was Tween-albumin medium.

Resistant Strains.—A resistant strain can be defined as one requiring a higher drug concentration to inhibit growth than did pre-treatment

TABLE III

ANALYSIS OF VARIANCE OF BATCH DIFFERENCES IN SOLID AND LIQUID MEDIUM TESTS OF PRE-TREATMENT STRAINS

	Sum of Square	Degrees of Freedom	Mean Square	Variance Ratio	P
Solid medium tests:					
Between batches	11·88	8	1·485	2·9	0·05–0·01
Error	19·10	37	0·516		
Liquid medium tests:					
Between batches	8·34	11	0·758	0·7	0·2
Error	34·99	34	1·031		

strains from the same patients. In theory, therefore, one should carry out sensitivity tests on a number of pre-treatment strains from the patients to obtain an estimate of the variation of the tests, and one should then see whether the sensitivity of the resistant strain lies outside the distribution of the sensitivities of these pre-treatment strains. However, it is sufficient to use the distribution of single tests on pre-treatment strains from different patients, since it has been shown that all these strains had the same degree of sensitivity.

The most important criterion of a method of performing sensitivity tests is its ability to detect resistant strains. This ability can be tested by first obtaining a definition of a resistant strain from the distribution of the sensitivities of pre-treatment strains and then, while using this definition, seeing how many out of a batch of strains with varying degrees of sensitivity are found to be resistant. Such a procedure was used to compare the efficiencies of liquid and solid medium tests, with readings taken after various periods of incubation and using either the "no-growth" or the "20 colonies" end-point in solid medium. The definition of resistance was obtained by fitting a normal curve to the results, by each method, of the tests on the pre-treatment strains (Group I). This curve was used to find the concentration of P.A.S. or the resistance ratio below which the sensitivities of 99 out of 100 would fail (standard deviation $\times 2.326$). Any strain whose minimal inhibitory concentration or resistance ratio was greater than this value was then defined as resistant. Using these definitions, the number of strains found to be resistant by each method was determined among the 85 strains that had previously been found resistant (Group II) or were suspected of possibly being resistant (Group III). The results are shown in Table IV.

The number of resistant strains that could be detected became larger as the period of incubation of the tests was increased. Thus, in liquid medium tests, 25 strains defined as resistant by their resistance ratios were detected after 10 days' incubation and 29 after 14 days. Similarly, on solid medium, using the "20 colonies" end-point, 19, 27, 30, and 31 resistant strains were found after, respectively, 14, 21, 28, and 42 days' incubation. A reading at 28 days on solid medium is probably the optimal period of incubation, because there was little gain in efficiency in increasing the period of incubation from 28 to 42 days and because of the desirability of an early report.

An examination of the results of 14-day readings in liquid medium and 28-day readings on solid medium shows that about the same number of

TABLE IV

DEFINITIONS OF RESISTANCE AND NUMBERS OF GROUPS II AND III STRAINS FOUND RESISTANT ACCORDING TO METHOD OF TESTING

	End-point	Period of Incubation (Days)	Lowest Result Indicating Resistance		No. of Strains in Groups II and III		
			Minimal Inhibitory Concentration (μg./ml. NaP.A.S.)	Resistance Ratio	Unreadable*	Sensitive	Resistant
Liquid	No growth	10	2		6	56	23
,,	,,	14	4		1	55	29
,,	,,	10		8	6	54	25
,,	,,	14		8	1	55	29
Solid	,,	14	8		3	59	23
,,	,,	21	16		2	58	25
,,	,,	28	16		1	54	30
,,	,,	42	16		0	52	33
,,	,,	14		16	3	62	20
,,	,,	21		16	2	60	23
,,	,,	28		8	1	54	30
,,	,,	42		8	0	54	31
,,	20 colonies	14	8		4	62	19
,,	,,	21	8		2	56	27
,,	,,	28	8		1	54	30
,,	,,	42	16		0	54	31
,,	,,	14		8	22	42	21
,,	,,	21		8	2	55	28
,,	,,	28		4	1	52	32
,,	,,	42		8	0	54	31

* No growth in control tube.

resistant strains were found, irrespective of the end-point used or whether the results were expressed in terms of the minimal inhibitory concentrations or of resistance ratios. There was an apparent slight advantage to solid medium tests, since they detected 30 to 32 resistant strains, whereas only 29 were detected by liquid medium tests. However, five of the seven strains that were resistant on solid medium but sensitive in liquid medium yielded resistance ratios of 4 in liquid medium, a result sufficiently high to suggest the necessity of retesting. On the other hand, only two of the six strains that were resistant in liquid medium, but not on solid medium, yielded resistance ratios on solid medium over 1; the values of their ratios were 2 and 4. Thus, if we take into account not only those strains definitely found resistant but also those with results on the borderline between sensitivity and resistance, it can be concluded that solid and liquid medium tests were about equally efficient in detecting resistant strains.

Only four of the Group III strains were found to be resistant in the parallel tests. Of these, three had resistance ratios of 2 or 4 in the original liquid medium tests and were resistant in at least two readings of the parallel tests. The fourth strain was just resistant in one reading on solid medium, but was sensitive in all other readings, and should probably be classified as sensitive.

The degrees of resistance found among the 36 strains that were resistant in liquid and on solid medium tests, or in either alone, are shown in Table V. The distribution of these strains according to their degrees of resistance was similar whichever method of testing was used. The resistance ratios in liquid and solid medium tests were in agreement with 21 strains, where agreement means that the ratio in one medium was not more than four times the ratio in the other. However, there was disagreement with 15 strains, seven yielding a higher result in liquid medium and eight on solid medium. Some of these discrepancies were very large: for instance, a strain with resistance ratios of 64 in liquid medium and 1 on solid medium and another with resistance ratios of 4 in liquid medium and more than 64 on solid medium. It can be concluded that individual determinations of the resistance ratios of resistant strains are frequently very inaccurate.

TABLE V

DEGREES OF RESISTANCE IN 36 STRAINS TESTED IN LIQUID AND ON SOLID MEDIUM

Resistance Ratio	No. of Strains	
	Liquid Medium*	Solid Medium*
>64	12	15
64	2	4
32	4	0
16	3	3
8	8	6
4	6	4
2	0	1
1–½	1	3

* Liquid medium tests were read after 14 days' incubation and solid medium tests after 28 days' incubation using the "20 colonies" end-point.

Stability of Resistant Strains.—Liquid medium tests were done twice on 32 strains that were found resistant in one of the two tests. The period between these tests varied from one to six months, during which the strains were subcultivated at least three times. A comparison of the resistance ratios, read after 14 days, shows agreement between the results of the two determinations in 19 strains and disagreement in 13 strains. In all cases of disagreement, the ratio at the repeat test was lower than the original value, and in No. 6 strain, originally found resistant, the repeat test indicated a sensitive result.

This loss of resistance was investigated in a further experiment in which nine strains, which had been found resistant in at least one previous test, were subcultivated every 10 days in Tween-albumin medium. Sensitivity tests on solid medium were set up at the start and from the third, sixth, and ninth subcultures. The results, shown in Table VI, fail to demonstrate any general tendency towards a decrease in the degree of resistance during the period of subcultivation.

TABLE VI

STABILITY OF RESISTANT STRAINS DURING SUBCULTIVATION IN TWEEN-ALBUMIN MEDIUM

Strain	Resistance Ratio after Successive Subcultivations			
	No. of Subcultures			
	0	3rd	6th	9th
1	1	1	1	2
2	1	1	1	2
3	1	4	1	8
4	8	4	64	16
5	32	8	64	16
6	32	16	16	32
7	128	>64	128	>128
8	128	4	32	32
9	>128	32	256	64

The apparent discrepancy between these results might be accounted for in two ways. It is possible that only a proportion of resistant strains are unstable. These unstable strains would tend to become less resistant during the first few subcultures in artificial media, thus accounting for the results of the original and repeat tests. On the other hand, those strains which were resistant at the beginning of the subcultivation experiment would be stable since they had retained their resistance until then. An alternative explanation is that some strains contain only a small proportion of resistant bacilli. During storage between the original and repeat tests these bacilli might have died out, with a resulting loss of resistance. Such an occurrence would be less likely during successive subcultivations at 10-day intervals.

Inoculum Size.—The effect of altering the size of the inoculum in the liquid and solid medium

TABLE VII

EFFECT OF ALTERATIONS OF INOCULUM SIZE ON MINIMAL INHIBITORY CONCENTRATION OF P.A.S. USING H37Rv AND A RESISTANT STRAIN

Strain	Volume of Inoculum (ml.)	Minimal Inhibitory Concentration of Na P.A.S. (μg./ml.)					
		Solid Medium Test*			Liquid Medium Test		
		Period of Incubation (Days)			Period of Incubation (Days)		
		14	21	28	14	21	28
H37Rv	0·01	4	8	32	8	16	32
	0·002	0·12	0·5	1	0·5	2	2
	0·0004	0·12	0·5	1	0·25	0·5	1
	0·00008	<0·12	0·12	0·5	<0·12	0·5	1
129	0·01	16	64	>128	16	16	16
	0·002	2	8	16	4	4	8
	0·0004	1	8	8	2	4	4
	0·0008	0·5	4	8	NG†	0·5	2

* 20 colonies end-point used. † NG = no growth in control tube of test.

tests was investigated using H37Rv and six resistant strains. Cultures grown in Tween-albumin medium were concentrated or diluted so that the bacilli which would be contained in volumes ranging from 0.01 to 0.00008 ml. of a 10-day growth were added in the drop or loopful used as the inoculum. Typical results are shown in Table VII. Alterations in the inoculum size had a marked effect on the minimal inhibitory concentrations of P.A.S. This effect was usually least between the inoculum sizes of 0.002 ml., the normal volume used, and 0.0004 ml. Thus it is most important to standardize the inoculum in P.A.S. sensitivity tests, although small decreases from the normal size are unlikely to cause serious error.

Discussion

For routine purposes two types of indirect sensitivity test have been suggested. In one of these (Medical Research Council, 1953b) a rather large number of tubes containing closely graded drug concentrations are inoculated, and the result is expressed as a resistance ratio. In the other (American Trudeau Society, 1952; U.S. Veterans Administration, 1954), a few tubes containing widely spaced drug concentrations are inoculated and, on the basis of experience with sensitive strains, growth on the medium containing a particular drug concentration is said to indicate resistance. Although the former method requires a larger number of tubes, the expression of the result as a resistance ratio has certain advantages. We have found that the end-point in P.A.S. sensitivity tests varied markedly according to the size of the inoculum, and to some extent according to the batch of solid medium prepared in the same laboratory. In spite of every effort to standardize the test, there are likely to be small variations in the inoculum used and in the preparation of the medium from one laboratory to another. These variations would be unimportant if a resistance ratio were obtained, since they would affect the titration of the standard sensitive strain to the same extent as the test strain.

We have shown that there was little difference between solid and liquid medium tests in the efficiency with which they detect resistant strains. It is probably slightly easier to prepare a wide range of drug concentrations in liquid than in solid medium. On the other hand, the solid medium test dispenses with the need for the preliminary subcultivation employed in the liquid medium test, during which contamination of the strains may occur. The time between obtaining a positive culture and reading the test is about the same for both methods, namely a month. Testing the P.A.S. sensitivity of isoniazid-resistant strains in Tween-albumin medium presents a problem, since these strains may grow poorly in this medium (Mitchison, 1953). However, modification of the medium by the addition of iron, keto-acids, haemin, or catalase (Cohn, Kovitz, Oda, and Middlebrook, 1954) may be sufficient to allow satisfactory growth of these strains. If solid medium tests are to be used, it is suggested that a suitable range of concentrations of Na P.A.S. would be 0.25, 0.5, 1, 2, 4, and 8 μg./ml. with one higher concentration, say 100 μg./ml. A resistance ratio for sensitive and slightly resistant strains could then be obtained. The results of the test should be read, using the "20 colonies" end-point, after four weeks' incubation, though readings at two and three weeks might be of value in the early detection of resistant strains. A resistance ratio of 8 would definitely indicate resistance and a ratio of 4 would be sufficiently suspicious to necessitate retesting the strain or others from the same patient.

Smaller laboratories might think it necessary to sacrifice the safeguards provided by the use of resistance ratio in favour of a test with a smaller number of tubes. In this case solid medium slopes containing 4 and 10 μg./ml. Na P.A.S. (approximately 3 and 7.5 μg./ml. P.A.S.) would probably be the most satisfactory. Our observation that approximately 10% of sensitive strains yielded some growth on slopes containing 4 μg./ml. Na P.A.S. after six weeks' incubation could be used as a check against variations in techniques. Thus readings at six weeks could be taken on the 4 μg./ml. slopes inoculated with the standard sensitive strains and, over a period, approximately 10% of them should show some growth. If growth were to occur on a smaller percentage or none of these slopes then some resistant strains would have been classified as sensitive. On the other hand if a larger percentage were to show growth then some sensitive strains would have been classified as resistant.

A laboratory subcommittee of the U.S. Veterans Administration (1954) has recently recommended that indirect P.A.S. sensitivity tests should be carried out by subculturing on to egg medium slopes containing 0, 10, and 100 μg./ml. P.A.S. Strains were to be considered resistant when the growth on the slope containing 10 μg./ml. P.A.S. was as good as on the control slope. If such a definition of resistance had been

employed in our test at least nine out of 30 resistant strains would have been misclassified as sensitive, since none of these nine strains yielded 20 or more colonies on slopes containing 16 μg./ml. Na P.A.S. (approximately equal to 10 μg./ml. P.A.S.).

Since six out of 32 strains reverted from resistance to sensitivity during storage, it would seem important to carry out sensitivity tests on primary cultures as soon as they have grown. Furthermore, nearly 20% of resistant strains tested both on solid and in liquid medium tests yielded sensitive results in one of these tests. Thus a sensitive result on a single strain from a patient cannot be relied upon. Since the presence of a P.A.S.-resistant strain is a contraindication to the use of chemotherapy with streptomycin and P.A.S. or isoniazid and P.A.S., it would be advisable to carry out two or preferably three tests on strains from every patient before the start of treatment.

Summary

Sensitivity tests to P.A.S. were carried out in Tween-albumin liquid medium and on slopes of Löwenstein-Jensen medium. Distributions of the sensitivities of 45 pre-treatment strains of tubercle bacilli, employing different methods, end-points, and periods of incubation, were obtained and used for defining resistant strains. All pre-treatment strains had the same degree of sensitivity ; apparent variations were due to the technical error of the tests, which was about twice that of similar tests for streptomycin sensitivity. The number of strains found to be resistant, out of a total of 35 strains previously found to be resistant or suspected of being resistant, was very similar whether the tests were carried out in liquid or on solid medium. Individual determinations of the degree of resistance were frequently very inaccurate. The minimal inhibitory concentration of P.A.S. was markedly affected by inoculum size and slightly by the method of preparing the solid medium. Some resistant strains reverted to sensitivity during storage.

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THE DETECTION OF STREPTOMYCIN RESISTANCE IN TUBERCLE BACILLI

BY

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It has been shown (Dye, Lynch, and Brees, 1953; Medical Research Council, 1953a and b; Pitts, Tempel, Miller, Sands, Fitzpatrick, and Weiser, 1953) that the treatment of tuberculosis with two drugs simultaneously markedly reduces the incidence of bacterial resistance provided that the organisms are fully sensitive to both drugs. If, however, the organisms are resistant to one of the drugs, resistance will develop to the second as quickly as if that drug were being given alone (Turnbull, Wallace, Stewart, and Crofton, 1953). Evidence obtained in this unit (to be published elsewhere) suggests that it is important to use a routine sensitivity test which will detect even low degrees of resistance. In Great Britain the generally accepted routine test for detecting streptomycin resistance in tubercle bacilli is that of serial dilution in Dubos and Davis liquid medium as recommended by the Medical Research Council (1953c). But in this unit we have had a number of patients excreting organisms which appeared to be sensitive by the routine test, although other factors in the cases suggested that streptomycin-resistant bacilli had emerged. In the present report an alternative method is described for the detection of streptomycin resistance in *Myco. tuberculosis* which appears to be more sensitive than the previous routine test.

Material

All strains of *Myco. tuberculosis* used were obtained from the sputa of patients with pulmonary tuberculosis. The sputa were homogenized by the alkali-acid or tri-basic sodium phosphate method, and cultured on Löwenstein-Jensen medium. The time between the inoculation of the culture and the testing for streptomycin resistance varied from four weeks to five months.

The cultures used are grouped as follows:

(1) Twenty-nine cultures from 29 patients who had never received streptomycin, to show the normal variation of the method.

(2) Thirty-four cultures which on the routine test appeared to be sensitive to streptomycin although they came from 22 patients whose organisms were suspected of being resistant for one or more of the following reasons: (*a*) Streptomycin was known to have been given previously alone or in combination with other drugs to which the patients' organisms were known to be resistant. (*b*) Isoniazid (*iso*-nicotinic acid hydrazide) resistance had developed during combined treatment with streptomycin and isoniazid. All of these patients had previously received streptomycin alone or in combination with *p*-aminosalicylic acid (P.A.S.), when their organisms had already become resistant to P.A.S. (*c*) The occurrence of apparent sensitivity to streptomycin in spite of the fact that on previous occasions resistance to the drug had been found by the routine test.

(3) Fifteen cultures from 15 patients from whom previous cultures had been shown to be resistant to streptomycin by the routine test to determine whether any of these cultures might appear sensitive by the new test.

Methods

The streptomycin sensitivity of the cultures was tested by the routine liquid medium method and by a solid medium method simultaneously. In both cases the standard H37Rv strain of *Myco. tuberculosis* was used as the control organism. The results were reported as a resistance ratio, that is the ratio of the minimum inhibitory concentration for the test organism to the minimum inhibitory concentration for the control strain.

Routine Liquid Medium Test.—The routine method was that recommended by the Medical Research Council (1953c) using Dubos and Davis' "Tween 80"-albumin medium (Dubos and Davis, 1946). The drug concentrations used were 8, 4, 2, 1, 0.5, 0.25, and 0.125 µg. per ml. of medium. The tubes were inoculated from a second subculture of the organisms in Dubos and Davis medium. All tests were read after 10 days' incubation and some were also read after 28 days.

Solid Medium Test.—The organisms were tested for resistance to streptomycin on Löwenstein-Jensen medium at concentrations of 64, 32, 16, 8, 4, 2, 1, and 0.5 µg./ml. These concentrations represent the actual

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amount of streptomycin added to the medium. After the addition of the streptomycin approximately 2 ml. quantities were dispensed into ½ oz. screw-capped bottles. These were inspissated in the horizontal position for one hour at 75 to 80° C. A suspension of a loopful of the growth of the primary culture of the organism was made in 0.3 ml. sterile distilled water by shaking with glass beads in a mechanical shaker. Using a nichrome wire loop of 3 mm. diameter, a loopful of the suspension was streaked up the centre of the slope. The slopes were incubated at 37° C. Readings were made at three, four, five, and six weeks. Growth was considered to be inhibited by the drug if fewer than 20 colonies appeared on the slope.

Solid Medium Test from the Liquid Medium Culture.—In order to ascertain whether resistance detected by the solid medium test but not by the liquid medium method was due to a failure of the resistant organisms to grow in the liquid medium subculture used for the routine test, a series of tests on solid medium was carried out using a loopful of the liquid medium subculture instead of the suspension. The number of organisms present in these two inocula is approximately the same.

Liquid Medium Tests without "Tween 80."—In order to ascertain whether the failure to detect resistance was due to the presence in the routine medium of "Tween 80," a series of tests was carried out in medium which did not contain any of this substance. The method used for these tests was exactly the same as for the routine test in all other respects. The liquid medium subculture from which the tests were inoculated contained "Tween 80."

Results

Cultures from Patients Who Had Never Received Streptomycin.—Table I shows the three and four weeks' readings of the solid medium tests on the 29 pre-treatment cultures. The results are expressed as resistance ratios. It will be seen that

TABLE I

NORMAL DISTRIBUTION OF RESISTANCE RATIOS ON SOLID MEDIUM TEST AFTER THREE AND FOUR WEEKS' INCUBATION

Incubation Period	No. of Cultures Showing Resistance Ratio						Total
	¼	½	1	2	4		
3 weeks ..	3	4	9	9	3	1	29
4 „ ..	0	4	11	8	5	1	29

at three weeks the readings fell within the range ¼ to 4. At four weeks this range was narrowed to ½ to 4. Results at five and six weeks were similar to those found on the four weeks' reading. The range of inhibition for the test organisms varied from 1 to 16 μg./ml. and for the standard H37Rv strain from 4 to 8 μg./ml.

From the normal distributions the standard deviation of the three weeks' readings is 1.217 and of the four weeks' readings is 1.013. The limiting resistance ratio for P=0.01 is 8 for the three weeks' results and 4 at four weeks. In view of these results it was considered that a four-week reading would be the most satisfactory to use.

The 29 pre-treatment cultures were also tested by the liquid medium method. The results of both methods, expressed as resistance ratios, are compared in Table II. By each method 28 of the 29 cultures tested gave a resistance ratio of 2 or less. By each method one strain gave a resistance

TABLE II

COMPARISON OF RESULTS OF LIQUID AND SOLID MEDIA TESTS ON 29 CULTURES FROM 29 PATIENTS WHO HAD NEVER RECEIVED STREPTOMYCIN

Solid Medium Test	Liquid Medium Test				Total
Resistance Ratio	Resistance Ratio				
	½	1	2	4	
¼	3	1	0	0	4
½	5	6	0	0	11
1	0	6	1	1	8
2	0	5	0	0	5
4	0	1	0	0	1
Total ..	8	19	1	1	29

"Resistance ratio"=the ratio of the minimum inhibitory concentration for the test organism to the minimum inhibitory concentration for the standard H37Rv strain.

The standard strain was inhibited by concentrations varying from 0.25 to 0.5 μg/ml. in the liquid medium and from 4 to 8 μg./ml. in the solid medium.

The liquid medium tests were read at 10 days and the solid medium tests at four weeks.

ratio of 4. Both the patients from whom these latter two strains were isolated have subsequently been treated with streptomycin in combination with another drug. In neither case has their subsequent progress suggested that the organisms were resistant to streptomycin.

By the routine liquid medium test the minimum inhibitory concentration for the test organisms varied from 0.125 to 1.0 μg./ml. and that of the standard H37Rv strain from 0.25 to 0.5 μg./ml. From the normal distributions of the 29 cultures also tested by the solid medium method, the standard deviation is 0.591 and the limiting resistance ratio for P=0.01 is just over 2. Utilizing a further 41 cultures tested by the liquid method only, the limiting resistance ratio is again just over 2. Since the tests are carried out with twofold differences between the concentrations, this means that the lowest resistance ratio giving a 99 to 1 probability of resistance is 4.

Although the numbers in these series are rather small for statistical analysis, the results suggest that by either method a resistance ratio of 4 will

only be obtained with one pre-treatment culture in 100. Therefore a ratio of 4 by either method has been taken as indicative of the emergence of a resistant strain.

Cultures Appearing Sensitive by Routine Test although Suspected of Resistance.—Table III gives the results of the solid medium test on 34 cultures which were sensitive on the routine liquid medium test. In every patient from whom these cultures were obtained streptomycin resistance was suspected for the reasons shown. It will be seen that in only one culture was a sensitive result found on the solid medium test. Of the remaining 33 cultures, 10 gave a resistance ratio of 4, nine of 8, seven of 16, and seven of greater than 16.

TABLE III

RESULTS OF SOLID MEDIUM TESTS ON 34 CULTURES FROM 22 PATIENTS WITH SUSPECTED STREPTOMYCIN RESISTANCE ALTHOUGH SENSITIVE BY ROUTINE TEST

Reason for Suspecting Streptomycin Resistance	No. of Patients	No. of Cultures	Solid Medium Test: Resistance Ratio 1	2	4	8	16	>16
Previous unsatisfactory chemotherapy ..	9	13	0	0	5	1	5	2
Later resistance to second drug ..	3	5	1	0	2	1	0	1
Resistance previously shown by liquid medium test	10	16	0	0	3	7	2	4
Totals ..	22	34	1	0	10	9	7	7

Cultures Resistant by the Routine Test.—A comparison of the degree of streptomycin resistance found by the liquid and solid medium tests on cultures resistant by both methods is shown in Table IV. It will be seen that all the cultures showing resistance by the routine test also showed resistance when tested on the solid medium.

TABLE IV

COMPARISON OF DEGREE OF RESISTANCE BY LIQUID AND SOLID MEDIUM TESTS ON 15 CULTURES OF KNOWN RESISTANCE BY LIQUID MEDIUM TEST

Resistance Ratio in Solid Medium Test	Resistance Ratio in Liquid Medium Test: 4	8	16	>16	Total
4	0	0	0	0	0
8	2	0	0	0	2
16	0	0	0	0	0
>16	3	2	2	6	13
Total	5	2	2	6	15

Cultures from Patients not Expected to Yield Streptomycin-resistant Organisms.—Twenty-four cultures, from patients in whom there was no reason to suspect the emergence of resistance, were all found to be sensitive by both methods.

Effect of Prolonged Incubation on Results of Liquid Medium Tests.—Table V shows the results of the liquid medium test after 10 and 28 days' incubation on 19 cultures which were resistant on the solid medium. It will be seen that in no case was the resistance ratio at 28 days more than twice that found at 10 days. No culture that gave a sensitive reading at 10 days appeared to be resistant at 28 days.

TABLE V

RESULTS OF LIQUID MEDIUM TEST AFTER 10 AND 28 DAYS' INCUBATION ON 19 KNOWN RESISTANT CULTURES

	28 Days' Reading Resistance Ratio	10 Days' Reading Resistance Ratio: Sensitive ¼	½	1	2	Resistant 4	8	16	>16	Total
Sensitive	¼	1	2	1	0	0	0	0	0	4
	½	0	1	1	3	0	0	0	0	5
	1	0	0	0	5	0	0	0	0	5
Resistant	4	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	1	0	0	0	1
	16	0	0	0	0	0	0	0	0	0
	>16	0	0	0	0	0	0	0	4	4
	Total	1	3	2	8	1	0	0	4	19

Effect of "Tween 80" on Results of Liquid Medium Tests.—A comparison of the liquid medium tests carried out with and without "Tween 80" on 27 cultures that were resistant to streptomycin on the solid medium is shown in Table VI.

TABLE VI

RESULTS OF TESTS IN LIQUID MEDIUM WITH AND WITHOUT "TWEEN 80" ON 27 KNOWN RESISTANT CULTURES BY SOLID MEDIUM TEST

	Resistance Ratio in Liquid Medium Without "Tween 80"	Resistance Ratio in Liquid Medium with "Tween 80": Sensitive 1	2	Resistant 4	8	16	>16	Total
Sensitive	1	0	0	0	0	0	0	0
	2	2	0	0	0	0	0	2
Resistant	4	2	7	1	0	0	0	10
	8	1	1	2	0	0	0	4
	16	0	1	0	0	0	0	1
	>16	0	1	3	1	1	4	10
	Total ..	5	10	6	1	1	4	27

Assuming that a resistance ratio of 4 indicates resistance by either method, it will be seen that of the 27 cultures tested two appeared to be sensitive when "Tween 80" was absent from the medium and 15 when "Tween 80" was present.

Comparison of Results of Solid Medium Test Inoculated from a Suspension of Primary Culture or Liquid Medium Subculture.—Table VII gives a comparison of the degrees of resistance found on solid medium tests inoculated from a suspension of the primary culture or from the subculture in the liquid medium. It will be seen that 37 of the 38 cultures were resistant by both methods. From only one culture was a sensitive result obtained from the liquid medium subculture, where a resistant result was found on the direct test.

TABLE VII

RESULTS OF SOLID MEDIUM TESTS ON 38 CULTURES ON A SUSPENSION OF PRIMARY CULTURE AND LIQUID MEDIUM SUBCULTURE

Resistance Ratio in Direct Test	Resistance Ratio in Test from Liquid Medium Subculture						Total
	1	2	4	8	16	>16	
4	1	0	2	3	1	1	8
8	0	0	3	2	2	1	8
16	0	0	2	2	2	0	6
>16	0	0	0	1	3	12	16
Total ..	1	0	7	8	8	14	38

Incidence of Resistance to P.A.S. and Isoniazid.—Of the 33 strains resistant to streptomycin on the solid medium test but not by the liquid medium method, 25 were resistant to P.A.S. and 22 to isoniazid.

Discussion

It is now well known that to use streptomycin in combination with isoniazid or P.A.S. in the treatment of tuberculosis only reduces the incidence of bacterial resistance to the second drug if the organisms are fully sensitive to streptomycin. It was therefore a little disturbing to find that in a number of cases the organisms appeared sensitive by the accepted routine liquid medium test, although other factors suggested that the organisms had developed streptomycin resistance. The value of streptomycin therapy in these cases was doubtful. A solid medium test was therefore tried in order to determine whether or not these cultures were in fact resistant to streptomycin. The results of these investigations are reported in this communication.

From the normal distribution curve of 29 cultures from patients who had never received streptomycin, it appears that in only one case in a hundred would a resistance ratio of 4 be found by the solid medium method. It therefore seems reasonable that for clinical practice a reading of 4 or above should be taken as indicating the development of resistance.

It was suggested by the Medical Research Council (1953c) that by the liquid medium test a resistance ratio of 8 was the lowest indicating resistance. The results of the present series suggest that a ratio of 4 is indicative of resistance 99 times out of 100. In fact the limiting resistance ratio is only just above 2 and if a narrower bracketing were used for the streptomycin concentrations a lower level might be found to be significant.

Tests on both solid and liquid medium were made on 34 cultures from patients in whom the existence of resistant tubercle bacilli was suspected either because of previous unsatisfactory chemotherapy, or because of resistance having been known to have been present on a previous occasion, or because resistance developed to a second drug during treatment with that drug and streptomycin in doses known to prevent the emergence of drug-resistant bacilli. All these cultures were sensitive by the liquid medium test. On solid medium 33 gave a reading of 4 or above, 23 of those being 8 or over. On only one culture from a case where there was reason to suspect the presence of streptomycin resistance was a sensitive result obtained on solid medium. Therefore in cases where resistance was shown by the solid medium method there was other evidence that resistance had developed. These results are in agreement with those reported by Holt and Cruickshank (1949) although they inoculated the solid medium tubes direct from the sputum concentrate.

All of the 15 cultures which showed resistance by the liquid medium test also appeared resistant by the solid medium test. It would therefore seem unlikely that by using the solid medium method a culture would be reported as sensitive which would have been resistant by the routine method.

The solid medium was not used as a screening method, and therefore there was less chance of detecting cultures resistant on the liquid medium but sensitive on the solid medium test. But of 24 cultures from patients in whom there was no reason to suspect the emergence of streptomycin resistance all were sensitive to the drug by both methods.

The question arises as to why there is this difference in the results of the two methods. It has been suggested that the detection of resistance by the solid medium test is due to the longer incubation period which allows of the appearance of the organisms with a slower growth rate. Results of the prolonged incubation of the liquid medium test do not support this theory. Of the 19

cultures tested, no culture giving a sensitive reading at 10 days appeared resistant after 28 days.

It has previously been found that the presence of "Tween 80" in the liquid medium enhances the inhibitory activity of streptomycin. Fisher (1948a) showed that of 20 strains tested in two types of liquid medium, 25% were resistant in Dubos and Davis' medium containing "Tween 80" whereas 55% were resistant when tested in Youmans medium where no "Tween" was used. This was also shown by testing strains for streptomycin sensitivity in a basal medium with the addition of "Tween 80" and of albumin singly and together (Fisher, 1948b). It was suggested that the use of 0.3% albumin in the Dubos and Davis' medium was sufficient to inactivate the "Tween 80" (Medical Research Council, 1948). In the present series only two of the 27 cultures resistant on solid medium were sensitive in the absence of "Tween 80," while 15 were sensitive when "Tween" was present, in spite of the addition of 0.3% of bovine albumin to the medium. Further work on this is being carried out.

It was possible that the differences in the results obtained in the liquid and solid media were due to a failure of the resistant organisms to grow in the liquid medium subculture from which the routine tests were inoculated. But resistance was demonstrated in 37 out of 38 cases where solid medium tests were inoculated from these subcultures.

The main disadvantage in using the Löwenstein-Jensen medium is the alleged breakdown of the streptomycin during inspissation. This might result in sensitive strains appearing resistant if a fixed minimum inhibitory concentration is used as indication of resistance. But the problem is largely overcome if the results are expressed as resistance ratios.

Of the 33 strains resistant to streptomycin on the solid medium but sensitive when tested in liquid medium, 25 were resistant to P.A.S. and 22 to isoniazid. The differences between the results of the streptomycin sensitivity tests by the two methods do not therefore seem to be related to resistance to either of these drugs.

In conclusion, it would seem that the solid medium test described will detect streptomycin resistance in tubercle bacilli more readily than will the routine liquid medium test.

Summary

A method is described for testing the streptomycin sensitivity of tubercle bacilli in Löwenstein-Jensen medium. The results are compared with those obtained in Dubos and Davis' liquid medium.

It is concluded that a resistance ratio of 4 by either method indicates resistance. Using this criterion, 33 of the 34 cultures tested from patients whose organisms were suspected of being resistant, were resistant by the solid medium test, although none were resistant by the liquid medium test.

There was no evidence that the solid medium test failed to detect resistance in cultures resistant by the liquid medium test.

The differences in the results of the two methods were not accounted for by the prolonged incubation of the solid medium test nor by the failure of the resistant organisms to grow in the liquid medium subculture.

The failure to demonstrate streptomycin resistance in the liquid medium may be due to the presence of "Tween 80."

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A NEW METHOD OF DETERMINING THE SENSITIVITY OF *M. TUBERCULOSIS* TO CHEMOTHERAPEUTIC AGENTS

BY

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Many methods are available for determining the sensitivity of *M. tuberculosis* to various chemotherapeutic agents. These may be divided broadly into three groups. In the first group the streptomycin is incorporated into solid media, such as that of Herrold (1931) or of Löwenstein (1930) and Jensen (1932) (Pyle, 1947; Johnson, 1950; Youmans, Ibrahim, Sweany, and Sweany, 1950; Tinne and Henderson, 1950; Wildy, 1951). The techniques included in the second group necessitate the use of liquid semi-synthetic media such as that of Proskauer and Beck (1894) as modified by Youmans (1944), or the medium of Long (1919) as modified by Dubos (1945) and Dubos and Davis (1946) (Medical Research Council report, 1948; Vollum, 1952). The methods of the third group of workers have not gained much popularity, as they present numerous technical difficulties. Emmart (1945) used chick embryos, while Cummings and Drummond (1949) recommended a slide culture technique. Only those in the first group can be considered practicable for use in the laboratory where there is a shortage of technical staff. The method to be described (modified from Beemer, 1951) is designed to fulfil this need.

Method

Preparation of Medium. — Löwenstein-Jensen medium is prepared as recommended by Jensen (1932) and poured into 3½-in. petri dishes, 35 ml. of medium being required for each dish.

The petri dishes are then inspissated at 80° C. for one hour on the first day and for a similar period at 75° C. on the second day. The water of condensation which collects on the lids of the petri dishes must be removed carefully by tilting the lids while the dishes are in the inspissator.

Finally the dishes are incubated at 37° C. overnight. This dries the medium suitably and any contaminated dish can be discarded. (With attention to the few precautions and details mentioned, such contamination is very rare.)

From the same batch of medium ordinary Löwenstein-Jensen slopes are prepared in screw-cap McCartney bottles to be used as the controls referred to later.

Preparation of Streptomycin-Liquid Paraffin Mixture.—One gram of streptomycin is dissolved in 2 ml. of sterile M/15 phosphate buffer of *p*H 7.8 and added to 78 ml. of liquid paraffin, giving a concentration of 1 in 80 streptomycin.

Procedure.—Every petri dish is inoculated with four strains of *M. tuberculosis*, of which one is H37Rv. A sterile throat swab is rubbed over Löwenstein-Jensen culture of the strain to be tested, and is then streaked across the medium in the petri dish. The

FIG. 1.—Löwenstein-Jensen plate with strip of filter paper impregnated with streptomycin. The three cultures show varying degrees of inhibition.

same swab is immediately inoculated on a fresh Löwenstein-Jensen slope as a control to make sure the test culture is still viable, and to compare the luxuriance of growth with that on the sensitivity plate.

A strip of sterile filter paper 3¼ in. × ½ in. is laid on the inoculated medium in the petri dish at right angles to the streaks of the organisms under test. The streptomycin-liquid paraffin emulsion (0.2 ml.) is evenly dispersed over the strip using a sterile Luer lock syringe fitted with a 17-g. Gurr-Sico hypodermic needle. An identical strip of sterile filter paper is then laid on the first to absorb the excess streptomycin-liquid paraffin emulsion, and the plate incubated at 37° C. for 10 days. To prevent drying, the plates are placed in a tightly lidded container with a small piece of damp gauze at the bottom.

Interpretation of Results.—After incubation confluent growth along the line of inoculation appears at a variable distance from the strip of filter paper (Fig. 1). The zones of inhibition are compared with that produced by the standard strain H37Rv. The distance between the edge of the filter paper and the rim of the petri dish at its diameter is arbitrarily divided into four equal parts. Each of these parts is designated with a plus symbol. Thus, if any strain is inhibited for one of these divisions, it is graded with 1 plus inhibition, and if for two divisions 2 plus, and so on. This inhibitory zone is then compared with that produced by the standard strain H37Rv, which usually shows a 2 to 3 plus inhibition. For example, if the test organism shows 1 plus inhibition, i.e., growth is inhibited for a distance equal to one-quarter the radius, and the control H37Rv shows 2 plus inhibition, the resistance of the test organism is reported as 2 × H. The length of the zone of inhibition depends on the amount of streptomycin which has diffused into the medium, and this may be affected by the physical properties of the medium and other factors. The quantitative expression of the results is therefore arbitrary, but is in reasonably close agreement with more strictly quantitative methods.

In some cases a few scattered colonies appear near the strip where confluent growth is inhibited by the streptomycin. In these instances the sensitivity of the strain of *M. tuberculosis* is determined as described above, but in addition the presence of the few resistant colonies is recorded (always provided that the control slope showed a confluent growth). It seems probable that this indicates the presence of resistant variants in a predominantly sensitive culture.

The table shows the results of sensitivity tests on 82 cultures by the method described and by the fluid culture method (Vollum, 1952). The results are identical in 26 cases and closely approximate in 36 cases. In no case are the results grossly divergent. For clinical purposes, the results of either test would be acceptable. Three strains which were insensitive by the plate method showed greatly increased resistance by the fluid culture method. The higher resistance found in one culture by the fluid method is shown by the plate method to be due to a few resistant colonies.

TABLE
COMPARATIVE RESULTS RECORDED IN TERMS OF RESISTANCE

Case Number	Fluid Method	Plate Method	Case Number	Fluid Method	Plate Method
3	1 H	1·5 H	69	1 H	0·5 H
4	2 H	2 H	70	1 H	1 H
5	1 H	2 H	73	2 H	2 H
7	1 H	1 H	76	1 H	1·5 H
9	1 H	1·5 H	78	1 H	1 H
10	1 H	1·5 H	80	0·5 H	1·5 H
12	2 H	1·5 H	83	1 H	1·5 H
13	4 H	1·5 H	84	0·5 H	2 H
14	20 H	Insensitive	87	2 H	1 H
17	2 H	1·5 H	90	1 H	1·5 H
18	2 H	1·5 H	91	0·5 H	2 H
19	1 H	1·5 H	92	1 H	1 H
20	2 H	1·5 H	93	1 H	1 H
21	2 H	1·5 H	95	0·5 H	2 H
23	1 H	1 H	96	0·5 H	2 H
24	4 H	1·5 H	97	1 H	1·5 H
25	1 H	1·5 H	98	2 H	1·5 H
26	1 H	1·5 H	99	1 H	1 H
33	0·5 H	2·5 H	100	1 H	1 H
35	1 H	1 H	102	0·25 H	1·5 H
36	0·5 H	2·5 H	103	1·5 H	1·5 H
37	1 H	1 H	104	2 H	1·5 H
38	1 H	1 H	105	1 H	1·5 H
39	1 H	1 H	106	2 H	2 H
41	1 H	1 H	107	2 H	1·5 H
42	1 H	1 H	109	10 H	Insensitive
44	5 H	2 H	110	0·5 H	2 H
45	1 H	1·5 H	111	0·5 H	1·5 H
46	1 H	1·5 H	112	10 H	Insensitive
47	1 H	1·5 H	113	2 H	2 H
51	1 H	1 H	114	0·5 H	1·5 H
52	1 H	1 H	115	0·25 H	2 H
53	0·5 H	1·5 H	*117	10 H	3 H
54	4 H	1·5 H	118	0·5 H	3 H
57	0·5 H	1·5 H	120	0·25 H	1·5 H
58	0·5 H	2 H	121	0·125 H	1·5 H
59	5 H	1 H	122	0·25 H	1 H
60	1 H	1 H	125	0·25 H	2 H
61	0·5 H	1·5 H	128	0·5 H	1·5 H
62	0·25 H	0·5 H	129	10 H	2·5 H
66	0·5 H	0·5 H	131	0·25 H	2 H

* Scanty colonies were present near strip.

Discussion

The simple plate technique described here has been shown to give results which are consistent and reproducible with the control culture H37Rv and which are in good agreement with those obtained by the serial dilution technique of Vollum (1952).

In our experience the method has a number of advantages which have prompted us to report our findings. Thus results are obtained relatively quickly and many cultures can be examined with the minimum of glassware and technical skill. Moreover, sensitivity can be assessed on a qualitative basis, and it is possible to see whether sensitive and insensitive organisms are present in the same culture.

Our thanks are due to Dr. R. L. Vollum for his advice and criticism; Dr. B. A. Dormer for making

available the necessary facilities for carrying out this investigation; Mr. O. F. Gibbs and Miss J. C. M. Holzhaus for invaluable technical assistance; and Mr. K. S. Mostert for the photographs.

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AN IMMOBILIZATION TEST FOR AMOEBIASIS

BY

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(RECEIVED FOR PUBLICATION NOVEMBER 16, 1954)

The demonstration of antibodies in syphilitic sera that will immobilize *Treponema pallidum* (Nelson and Mayer, 1949) led Cole and Kent (1953) to investigate and demonstrate that immune rabbit sera were capable of immobilizing *Entamoeba histolytica*, and to suggest that sera from human cases of amoebiasis might show the same phenomenon. Their rabbit sera did not show immobilizing activity to a high titre but good immobilization of amoebae at single and twofold dilutions: further dilution of the sera led to a considerable fall in the immobilizing activity. In preliminary studies with sera from human cases of amoebiasis, these authors tested 13 sera, five of which showed some immobilizing activity in low dilution. The immobilizing effect appeared to be specific because 48 normal sera were without effect upon the amoebae.

A further laboratory test for the diagnosis of amoebiasis would clearly be desirable owing to the unsatisfactory nature of the complement-fixation test. In this paper the results of applying an immobilization technique in parallel with a complement-fixation test are described.

Methods

Complement-fixation Test. — Antigens were prepared from washed concentrated suspensions of three-day-old *Entamoeba histolytica*, from which alcoholic extracts were made by the method of Weiss and Arnold (1934). Control antigens were similarly prepared using fluid from the culture slopes.

Hyperimmune rabbit sera were used as controls. Cultures of amoebae were washed three times and concentrated to one-tenth volume, being suspended in physiological saline. After mixing with penicillin, 100 units/ml., and streptomycin, 100 μg./ml., 0.5 ml. of the preparation was then inoculated intravenously into rabbits on alternate days, until a total of 10 injections had been given. The animals were bled after an interval of eight days.

Sera were inactivated at 56° C. for 30 minutes. A unit volume of 0.1 ml. of diluted serum was used for each tube and an equal volume of 2.5 M.H.D. of pooled guinea-pig complement was added, followed by 0.1 ml. of antigen. The total volume of each tube was made up to 0.8 ml. with saline. Fixation was carried out at 37° C. for one and half hours before adding 0.2 ml. sensitized sheep-cell system. This consisted of 5 M.H.D. haemolytic amboceptor in a 5% sheep-cell suspension. Tubes were placed in a 37° C. water-bath for 30 minutes before reading.

Preliminary tests had shown that 2.5 M.H.D. of complement with one and a half hours' fixation at 37° C. was superior to other periods of fixation and temperature. Particularly poor fixation was obtained at lower temperatures.

In the test, antigens were used at concentrations of 1 in 8 and 1 in 16 and antisera were usually tested at 1 in 4 and 1 in 8.

Immobilization Test.—Amoebae were cultivated on Dobell and Laidlaw's (1926) modification of Boeck and Drbohlav (1925) medium. Subcultures from the sediment at the bottom of the slopes were made every three days for maintenance. Forty-eight-hour cultures were used for the immobilization test. A modified medium, in which 50% horse serum was substituted for 1 in 8 serum, in an endeavour to obtain growth free of rice starch, was tried, but as the yield of amoebae was very poor the method was abandoned.

All glassware used in the experiment was cleaned with acid.

The sediments from 48-hour-slope cultures of *Entamoeba histolytica* were pooled and allowed to stand for 30 minutes, after which time most of the supernatant fluid, which contained scanty amoebae, was removed. A concentrate containing between 200,000 and 400,000 amoebae/ml. was used for the test. Equal volumes of the serum to be tested and the amoebic suspension were mixed in a tube, and a drop of the mixture was transferred to a microscope slide and sealed with a coverslip and vaseline; all materials used in these manœuvres had been previously heated to 37° C. The preparation, which was then maintained at 37° C. in the incubator, was examined at intervals under the 2/3 lens on a warmed stage, and the number of "rounded up" or immobile amoebae estimated. Twenty-five amoebae were counted and the result expressed as a percentage. "Non-rounded up," immobile amoebae were not regarded as immobilized, and the test was controlled by examining known positive and negative sera at the same time as the sera under test. The results were plotted as a curve, and sera showing more than 70%

immobilization in the first hour were regarded as positive, those showing between 70% and 30% immobilization were considered doubtful, and those showing less than 30% immobilization negative.

Results

Immobilization is maximal at 30 minutes, and thereafter in most instances motility is gradually restored. In addition positive sera, which have not been inactivated by heat to destroy complement, may show lysis as well as immobilization.

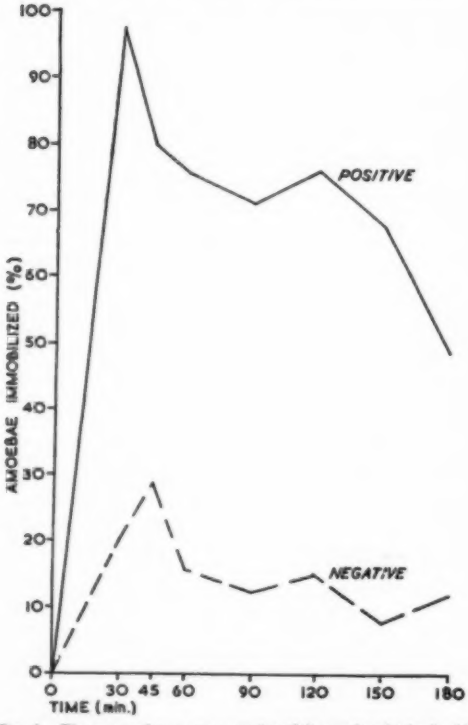

FIG. 1.—The curves shown are examples of the results obtained with typical positive and negative sera.

Of 25 sera tested, six were positive, three doubtful, and 16 negative by the immobilization test. The complement-fixation test was positive in three of the 25 sera tested, one of which was negative by the immobilization test. All sera tested were from active proved cases of amoebiasis save two, which were from old cases of amoebiasis with unproved but suspected recurrences. The latter sera were negative by both tests.

Fig. 1 illustrates typical findings with positive and negative sera.

Discussion

Immobilization of amoebae by human sera has been clearly demonstrated, but the very definite results such as were obtained by Cole and Kent (1953) with hyperimmune rabbit sera have not been obtained by us even in severe cases of human amoebiasis.

Amoebic dysentery has never been shown to be a disease stimulating a high level of antibody production in complement-fixation tests, and the interpretation of these tests has never been very satisfactory, as shown by Hussey and Brown (1950) and Weiss and Arnold (1934). Therefore, it is hardly surprising that this apparent lack of antigenicity is reflected in immobilization tests.

Heated inactivated sera showed immobilizing activity of a temporary nature, and the immobilized amoebae gradually regained their activity after approximately one hour. This is in accordance with the observations of Cole and Kent (1953). The action of complement is not required for this effect, and it has been shown that if complement is present lysis of the amoebae results. It is clear that immobilization tests are unlikely to be more satisfactory than complement-fixation tests owing to the considerable technical difficulties in maintaining the motility of the amoebae in the controls and also because such tests appear to be little more sensitive than the present complement-fixation test.

It has not been possible in the present study to elucidate the nature of the immobilization reaction, but it seems that it is unrelated and probably fundamentally different from the reaction involving the use of complement. This matter appears to merit further investigation.

Summary

An immobilization test for amoebiasis has been described, and a comparison made with the complement-fixation test. The immobilization test was found to be little superior to the complement-fixation test and involved considerably more technical difficulty.

Our thanks are due to Colonel W. R. M. Drew, C.B.E., for much encouragement and advice and to many pathologists who sent us specimens.

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PHOSPHATE CONTAMINATION OF COMMERCIAL HEPARIN

BY

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Heparin is widely used as an anticoagulant in biochemical laboratories. Hawk, Oser, and Summerson (1947) state that heparin is an excellent anticoagulant, as it does not alter the distribution of diffusible substances between cells and plasma. Jorpes (1946) states that heparin neither influences the volume of the blood corpuscles nor interferes with the chemical analysis. In the course of routine estimations of plasma inorganic phosphorus (P), it was noted that the heparin used was heavily contaminated with phosphate and was responsible for erroneously high values. We have not been able to find any report suggesting that heparinized blood is not suitable for the estimation of phosphate.

Materials and Methods

Phosphate Estimations.—These were carried out, using the method of Fiske and Subbarow (1925).

A crude preparation of heparin is normally used in this laboratory for the preparation of heparinized blood tubes. This preparation consists of short-chain molecules which are a by-product of the penultimate stage in the manufacture of therapeutic heparin. The preparation is equivalent to 30 units heparin per mg. For routine use 0.1 ml. of a solution of 5 g./165 ml. of this preparation is added to each 10 ml. blood tube and the tubes are heated in an oven at 37° C. to dryness. The pure preparation of therapeutic heparin made by the same manufacturer, which contains 99.8 units per mg., was also used. For purposes of comparison the five other commercial preparations of heparin available in the British Isles were obtained, using the solution of 1,000 units per ml.

Results

TABLE

Heparinized Plasma (mg. P/100 ml.)	Serum (mg. P/100 ml.)
7·7	4·0
6·3	3·8
8·1	3·4
7·7	3·8
5·4	3·7
7·3	4·2
5·9	3·7

It was consistently found that heparinized plasma had a higher P content than serum. The table shows a number of typical values.

The heparin solution was shown to be heavily contaminated with phosphate.

Discussion

An attempt was made to introduce a correction factor for the amount of phosphate contamination, but this was abandoned because (1) it would necessitate the accurate measurement of 0.1 ml. heparin solution by the unskilled women who prepare the tubes for blood; (2) the amount of blood added from the syringe in the ward could not be accurately measured without a great deal of difficulty, as it would entail the cooperation of the large number of doctors, students, and nurses who withdraw blood samples; (3) it appeared likely that the amount of the contamination might vary from batch to batch of heparin. It was considered that more reliable results would be obtained if estimations of P were carried out on serum rather than on heparinized plasma.

The therapeutic heparin preparation of the firm which supplied our heparin, and all the other preparations of heparin available, were shown to contain traces of P. These are not ordinarily used in this laboratory because of their high cost.

Four of the firms which manufacture heparin have informed us that they use phosphoric acid in the process, but in most cases steps to remove it are carried out. Nevertheless it seems very likely that at least traces of P may remain in the refined product. One firm which does not use phosphoric acid has informed us that during the autolysis of the starting tissue ammonia is added, and that ammonium magnesium phosphate is formed from magnesium and phosphorus present in the tissue. This tends to adhere to their stainless steel containers. They have also noticed that some batches of charcoal used in their purification process are contaminated with phosphate. Traces of P have

been found in their preparation. In either of these manufacturing processes P is likely to be present as a contaminant, and the actual amount may vary from batch to batch.

In these circumstances it seems highly undesirable to add heparin as an anticoagulant to blood on which an estimation of inorganic phosphorus is to be carried out.

Summary

Phosphorus has been found as a contaminant in heparin which was being used as an anticoagulant in a routine biochemical laboratory.

The processes employed in the commercial preparation of heparin are likely to lead to the contamination of the finished product with phosphate.

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AN ORTHO-TOLIDINE METHOD FOR THE DETECTION OF OCCULT BLOOD IN FAECES

BY

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Benzidine is still the most commonly used reagent for occult blood tests in faeces. About three years ago, however, the manufacture of AnalaR benzidine in this country was discontinued as it was shown that benzidine has very marked carcinogenic properties, particularly in some of the stages of purification to AnalaR specification. This did not appear to be widely recognized probably because most laboratories still had stocks of the AnalaR benzidine. We drew attention to this state of affairs two years ago (Kohn and O'Kelly, 1952). Since then it has been found that the benzidine available is not sufficiently reliable for occult blood testing even when various methods of purification were tried. This affects, of course, any benzidine method whatever the technique.

A review of other reagents which could replace benzidine showed that ortho-tolidine had been used both for urine and for faecal occult blood tests (Zwarenstein, 1949; Hawk, Oser, and Summerson, 1947; Caplan and Discombe, 1951). As far back as 1912 and 1913 Ruttan and Hardisty introduced an ortho-tolidine method for demonstrating occult blood in faeces and urine and regarded it as being superior to the benzidine method. Kiefer in 1934 again drew attention to the advantages of the method. O-Tolidine methods, however, did not become very popular, probably partly due to the misconception that they were too sensitive. Trials of various o-tolidine techniques showed that false positive results could be obtained, but on reinvestigating the conditions of the test, the properties of the reagent, and the proportions of its components it appeared that this "over-sensitivity" was due either to wrong proportions of the reagents employed or to the practice of filter paper methods, which tend to produce false results with any reagent. It was then found that the sensitivity of the ortho-tolidine reagent could be modified at will by varying either the concentrations or the proportions of the reagents taking part in the reaction (Figs. 1 and 2 and Table I).

TABLE I

EFFECT OF VARYING CONCENTRATIONS OF O-TOLIDINE ON OCCULT BLOOD TESTING

Concentration of Blood in 10% Faecal Suspension	Dilutions of Stock 4% Ortho-tolidine in 50% Glacial Acetic Acid				
	50%	33%	20%	10%	5%
1 : 1,000	+++	+++	+++	++	+
1 : 2,500	+++	+++	++	+	±
1 : 5,000	+++	++	+	−	−
1 : 10,000	+++	++	+		
1 : 20,000	++	+	±		
1 : 30,000	+	±	−		
1 : 50,000	+	−			

O-tolidine reagent: H_2O_2 ratio—4 drops to 1 (approximately 12 s.w.g. pipette).

+++ = Very strong positive, intense blue colour developing almost immediately.

++ = Strong positive, blue to bluish-green developing within 5 to 20 sec.

+ = Positive, greenish-blue to deep green developing within 1 min.

± = Weak positive, green to light green developing within 2 min.

33% corresponds to the concentration of the working solution recommended. As many of the factors involved (colour of the faeces, strength of faecal suspension, estimation of colour) are difficult to control the interpretations given in this table can only be approximate and are not intended to convey strictly quantitative data.

These investigations and further trials eventually led us to adopt a test-tube technique described below, which has been in routine use in this laboratory for the last three years, during which time over 2,500 examinations have been performed. The method was standardized so that it is slightly less sensitive than the benzidine test, using AnalaR benzidine and the standard textbook technique.

Method

Reagents.—The following are required:

Stock Solution.—A solution of 4% ortho-tolidine (AnalaR B.D.H.) in ethanol is made up which is stable for a very long time. At approximately 4° C. refrigerator temperature it partly precipitates, but this does not affect its efficiency. For practical reasons it was found best to distribute the stock solution in small quantities, e.g., 2 ml., into test-tubes, to which

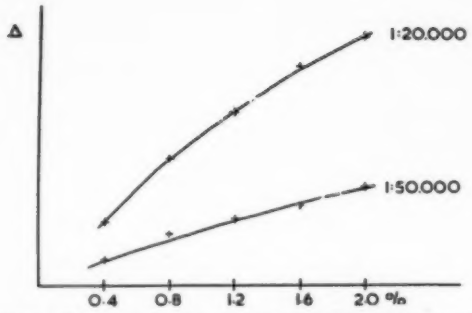

FIG. 1.—Effect of varying the concentration of o-tolidine in the reagent. Concentration plotted against optical density of the colour obtained with two dilutions of blood. One drop of blood-water dilution was added to a mixture of 4 drops of the o-tolidine solution and 1 drop of 20 vol. H_2O_2 (12 s.w.g. pipettes were used). After five minutes 4 ml. water was added and the optical density Δ measured in a Spekker absorptiometer.

the diluent is added when required to form the working solution. The precipitate then becomes completely dissolved.

Working Solution.—Equal parts of stock solution, glacial acetic acid and distilled water, which solution, once prepared, is stored at 4° C. in a refrigerator and is stable for at least a month.

20-Volume Hydrogen Peroxide.—Both the o-tolidine working solution and the hydrogen peroxide are best kept in bottles or tubes fitted with dropping pipettes in the stoppers. This ensures the delivery of constant (if not equal) volumes. T.K. and dropping bottles are not recommended as there may be a large variation in the size of the drops.

Technique.—A portion of faeces about the size of a pea is boiled in about 5 ml. of water. Boiling is carried out as, apart from the controversial subject of non-specific enzyme inactivation, it is the simplest way of obtaining a fairly homogeneous suspension. In our experience the boiling does not appear materially to affect the final result.

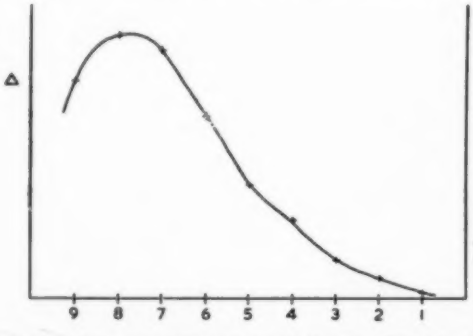

FIG. 2.—Effect of varying the o-tolidine : H_2O_2 ratio. Ratio plotted against optical density of colour obtained with a 1 in 10 dilution of blood. Figures on the horizontal axis denote number of drops of the o-tolidine working solution. H_2O_2 was added to each tube to make up a total of 10 drops (12 s.w.g. pipette). Otherwise procedure as in Fig. 1.

In a clean test-tube are placed 4 drops of the working solution followed by 1 drop of 20-vol. hydrogen peroxide. This is left standing for one minute to ensure that there is no contamination of tube or reagent, and 1 drop of the faecal suspension is added. This can be most easily delivered from the tip of the glass rod used to mix the faecal suspension, and also has the advantage of giving a fairly constant and reproducible volume.

Interpretation.—A positive result is indicated by the appearance of a green or blue colour developing within a minute and lasting from a few minutes to several hours, depending upon the intensity of the reaction. The colour develops more gradually and lasts much longer than with benzidine. With increasing concentration of blood the colour develops more quickly and changes progressively from green to deep blue. The amount of blood present can thus be assessed by the colour and by the speed of its development (Table I). We do not think, however, that any hard and fast interpretation scheme can be of much real value. The intake of iron salts or chlorophyll does not produce false results. Bromides and iodides give a colour change *in vitro*, but do not appear to interfere with faecal examinations.

Adaptation to Slide-test Technique.—A further attempt was made to adapt the o-tolidine method to the slide-test technique, if for reasons of expediency this is preferred. It was found that the o-tolidine reagents could be employed for this purpose with equally satisfactory results, provided certain precautions are observed. These consist first in using a water-repellent surface on which the faecal smears are made. This procedure prevents creeping at the edge of the drop of reagent. (The creeping thin layer of the reagent dries very rapidly and very often leads to the appearance of a blue ring which may give the impression of a false positive result.) Secondly, as in the slide technique undiluted faeces is used, a less sensitive reagent is required. (An average smear contains at least twice as much faecal material as a drop of faecal suspension.) The decreased sensitivity is achieved by altering the proportions of the reagent. The following method has been adopted and has given in our hands satisfactory and reliable results.

A cavity porcelain tile is treated with "repelcote" silicone (Midland Silicones Ltd.; distributed by Hopkin and Williams), or alternatively smeared thinly with some greasy substance, e.g., vaseline. A smear is then made in one of the cavities with a small amount of faeces on a glass rod, orange stick, or the tip of the examining glove. Then a drop of 20-vol. hydrogen peroxide is added to the smear followed by one drop of the o-tolidine working solution. A green or blue colour, depending on the intensity of the reaction,

developing within one minute indicates a positive result. The interpretation is similar to that of the test-tube technique.

The routine examinations for occult blood in faeces are performed on patients on a three-day controlled meat-free diet. We believe that this precaution is justified, but this paper is solely concerned with a matter of technique. The reagents can, however, be easily adjusted to the sensitivity of the Gregersen test if this is preferred.

Summary

Attention is drawn to the difficulties in obtaining pure and reliable benzidine for occult blood tests.

A simple method for the detection of occult blood in faeces is described. It has the following advantages :

(1) The working solution is stable and need not be prepared each time a test is performed.

(2) The volumes of the reagents and of the faecal suspension are measured and not guessed approximately, thus introducing a reasonable degree of accuracy.

(3) The method is flexible, as the sensitivity of the test can be modified according to one's particular views or requirements.

(4) AnalaR ortho-tolidine is easily obtainable and the method is economical as only small quantities of the reagents are used.

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THE ESTIMATION OF PROTEIN IN CEREBROSPINAL FLUID USING THE M.R.C. PHOTOMETER

BY

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Turbidometric methods are widely employed for the estimation of protein in cerebrospinal fluid (C.S.F.). Precipitation is generally effected using salicylsulphonic acid, and subsequent comparison made with standard reference tubes, e.g., of the formazin gelatin type (King, 1951a). Unfortunately these suffer "ageing," and matching in artificial light is often difficult.

The following technique has been employed in this laboratory for the past two years.

Method

To 1 ml. of C.S.F. in a 6 in. by $\frac{1}{2}$ in. test-tube is added 3 ml. of 3% aq. salicylsulphonic acid solution, mixed, and allowed to stand for five minutes. At the end of this time the turbid suspension is introduced into the right-hand cell of the photometer, using a distilled water blank in the other cell. Matching is as for a haemoglobin estimation (No. 2 eyepiece, Ilford yellow/green filter 625), and correction is for personal reading error using the standard wedge. The corrected reading is a direct measure of the concentration of protein in mg. per 100 ml. of fluid.

To test the efficiency of the method, 350 clear fluids were assayed for protein content by (*a*) direct comparison with standard tubes, (*b*) the photometric method described, (*c*) the micro-Kjeldahl method using SeO_2 (King, 1951b), and (*d*) the micro-Kjeldahl method followed by iodometric titration (Rappaport and Lasowski, 1943).

Comparison of the results of method (*a*) with (*c*) and (*d*) showed a mean standard deviation for the series of ± 5.2 mg., and for method (*b*) with (*c*) and (*d*) a mean standard deviation of ± 1.8 mg.

Further, comparison of methods (*a*) and (*b*) in a series of 2,500 specimens has confirmed that the photometric method always yields results of the same order, but reveals that the photometric method affords greater facility for detecting small changes in protein concentration in the C.S.F. when patients are being subjected to daily lumbar puncture.

Conclusion

It is considered that the M.R.C. Grey wedge photometer is an adequate instrument for estimating protein content of C.S.F.

It can also be used for those other turbidometric procedures which normally require comparison with standard protein reference tubes, e.g., the thymol turbidity test and the zinc sulphate test.

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COLOUR PRODUCTION AND STABILITY IN THE FOLIN AND WU METHOD OF BLOOD GLUCOSE ESTIMATION

BY

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The methods most commonly employed for the estimation of blood glucose are dependent upon the ability of glucose to reduce alkaline copper solutions. Reagents containing phosphomolybdic acid are then frequently used to form a blue complex by combination with the reduced copper. All these methods suffer the disadvantage that the final colour fades at such a rate as to imperil its accurate comparison with a standard glucose solution similarly treated.

Fading was neither very apparent nor of much importance when visual colorimeters were employed with direct comparison of standard and unknown colours. Folin (1929) first recorded the existence of fading and described a method to combat it. Interest was aroused by the general adoption of the photoelectric colorimeter, and many technical modifications were introduced to circumvent fading. These were remarkable for their variety rather than for their success in routine use.

In the present investigation the factors influencing fading have been determined and a new modification of the Folin and Wu technique has been evolved which simplifies the standard technique at the same time as it eliminates fading. Although the Folin and Wu (1920) method was chosen for this investigation the principles involved are applicable to other methods of blood glucose estimation in which similar reagents are used.

Reagents and Apparatus

Analar grade materials were used in the preparation of all reagents.

Copper Reagent.—This was made up as described by Folin and Wu (1920) but stored as two solutions as recommended by Folin (1929). Copper reagent was freshly prepared from the two constituent solutions each day.

Phosphomolybdic Reagent (P.M.R.).—This reagent was also prepared as described by Folin and Wu (1920).

Standard Glucose Solution.—This contained 2.5 mg. glucose per 100 ml. in saturated benzoic acid.

Standard Folin and Wu tubes of bulb capacity 4 ml., a special Folin and Wu tube of bulb capacity 20 ml., an E.E.L. portable photoelectric colorimeter, and a set of "large" colorimeter tubes were required.

The Effect of Dilution upon Colour Stability

Experiment 1 : Stability of Colour of Undiluted Mixture.—Previous workers (Folin, 1929 ; Lehmann and Silk, 1952) have noted that the amount and rate of fading are increased when the reduced copper-P.M.R. mixture is diluted with water. Fading of the undiluted mixture was compared with that found in an identical sample diluted with distilled water.

Technique.—Copper reagent, 10 ml., and 10 ml. of standard glucose solution were mixed in the special Folin and Wu tube. The tube was stoppered with non-absorbent cotton wool and heated in a boiling water-bath for exactly six minutes. The tube was immediately cooled to room temperature and after three minutes had elapsed 10 ml. of P.M.R. added. After a further two minutes the contents of the tube were thoroughly mixed and as much CO_2 as possible removed by agitation (one minute), then 3 ml. was pipetted into a large E.E.L. colorimeter tube containing 6 ml. of distilled water. The contents of the tube were thoroughly mixed and read in the colorimeter at 1, 10, 30, 60 minutes and 2, 4, and 22 hours after dilution. The density of the undiluted mixture was determined at similar times by making a fresh 1 in 3 dilution on each occasion with immediate mixing and reading. The colorimeter tubes were tightly stoppered with cotton wool and stored in the dark between reading, as was the large Folin and Wu tube. Environmental temperature was kept as constant as possible. The constancy of the colorimeter was

checked using a neutral grey solution giving a colorimeter reading similar to that of the solutions under test.

Fig. 1 illustrates the results obtained and shows that fading of the undiluted mixture was much less than that which followed dilution with distilled water.

Experiment 2: Dilutions of P.M.R. as Diluting Agent instead of Distilled Water.—Four dilutions of P.M.R. were prepared: $\frac{1}{6}$, $\frac{1}{3}$, $\frac{1}{2}$, $\frac{2}{3}$ strength in distilled water. These were used as diluting agents and fading compared with that found when distilled water was used for this purpose. The fading of the undiluted mixture was also determined.

Technique.—This was as described above, and 3 ml. quantities of the reduced copper-P.M.R. mixture were dispensed into a series of colorimeter tubes each containing 6 ml. of the diluents under test.

The results are shown in Fig. 2. The fading seen with $\frac{1}{3}$ strength P.M.R. as diluent was very similar to that of the undiluted mixture. The fading seen with $\frac{1}{6}$ strength P.M.R. was intermediate between $\frac{1}{3}$ strength and distilled water. Increased density of colour occurred with $\frac{1}{2}$ and $\frac{2}{3}$ strength reagent up to four hours, after which there was a slight decrease.

Experiment 3: Dilute Phosphoric Acid as Diluting Agent in Place of Distilled Water.—Lehmann and Silk (1952) have suggested that it is the alteration in phosphoric acid concentration which is responsible for fading on dilution. We have used as diluting agent dilutions of phosphoric acid equal to those found in the dilutions of P.M.R. used above.

Technique.—Dilutions of phosphoric acid containing 4.0, 8.0, 12.5, and 16.5% v/v of acid in distilled water were prepared and used in an identical manner to that described above.

The results shown in Fig. 3 are markedly different from those in Fig. 2. Fading was quite considerable with lower concentrations of acid and higher ones apparently caused a rapid immediate loss of colour. No concentration caused any increase in density of colour.

Experiment 4: Initial Addition of P.M.R. Made in Diluted Form.—Since the degree of fading

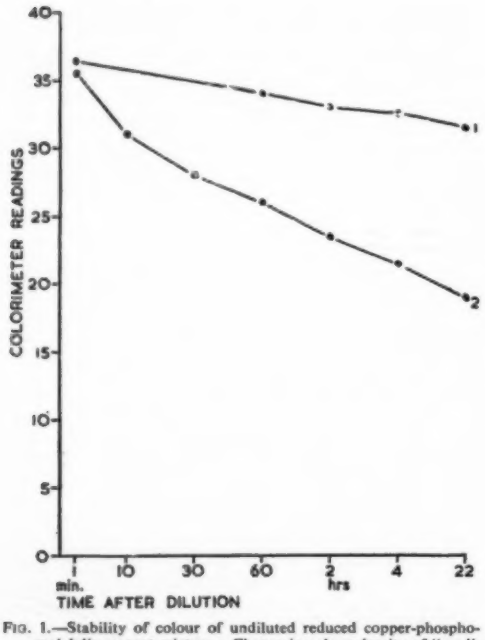

FIG. 1.—Stability of colour of undiluted reduced copper-phosphomolybdic reagent mixture. Change in colour density of "undiluted mixture" (1) compared with that on dilution with distilled water (2).

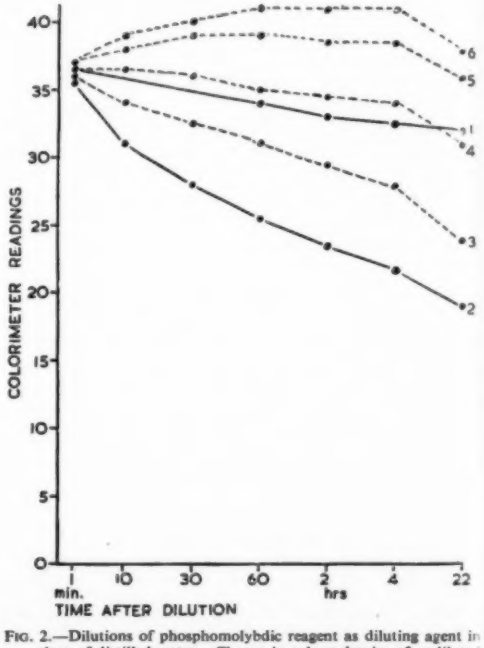

FIG. 2.—Dilutions of phosphomolybdic reagent as diluting agent in place of distilled water. Change in colour density of undiluted mixture (1) and that diluted with distilled water (2) compared with changes found when $\frac{1}{6}$ strength (3), $\frac{1}{3}$ strength (4), $\frac{1}{2}$ strength (5), and $\frac{2}{3}$ strength (6) reagent are used as diluents.

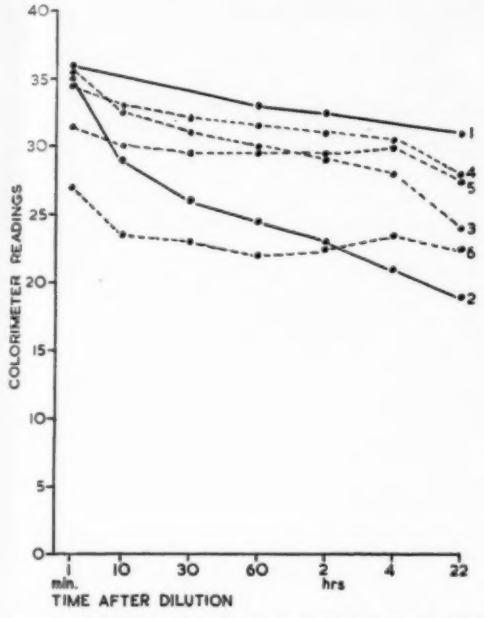

FIG. 3.—Dilute phosphoric acid as diluting agent in place of distilled water. Change in density of undiluted mixture (1) and that diluted with distilled water (2) compared with changes found when 4% (3), 8½ (4), 12.5% (5), and 16.5% (6) v/v of phosphoric acid are used as diluents.

seemed related to change in the concentration of the P.M.R. reagent the effect was determined of using the reagent already diluted.

Technique.—Dilutions of P.M.R. were prepared in distilled water, their strength ranging from 7% to 100%. Copper reagent, 2 ml., and 2 ml. of standard glucose solution were dispensed into a series of standard Folin and Wu tubes. After the contents had been mixed the tubes were stoppered with non-absorbent cotton wool and heated together in a boiling water-bath for exactly six minutes. After cooling to room temperature, the P.M.R. was added as a 14 ml. quantity of one of the dilutions, a different strength being used for each tube. After one minute the contents of the tubes were mixed and readings determined at times similar to those employed in the previous experiments, but, as changes detected in the first five minutes were minimal, first readings were made at four minutes for technical convenience.

The results are illustrated in Fig. 4. The uppermost point at each dilution represents density of colour at four minutes and the lowermost density at 22 hours. The line joining them represents fading during that period. It will be seen that as the concentration of P.M.R. increased so did

Final Concentration of Reagent (%)	Experiment 2 (Reagent Added in Two Stages)		Experiment 4 (Reagent Added in One Stage)	
	Concentration after 1st Addition 33%			
	Time after 2nd Addition		Time after Addition	
	1 min.	22 hr.	4 min.	22 hr.
11	35·5	19	25·5	18·5
22	36	24	30	24
33	36·5	31	34	31·5
44	37	36	39	37·5
55	37	38	41·5	37·5

the density of colour up to a maximum at about 60% final concentration; beyond this there was a fall. The amount of fading decreased up to 40% final concentration after which it increased again.

Experimental conditions were so arranged that the final concentration of reduced copper was exactly similar to that in Experiment 2 when the P.M.R. was added in two stages—first undiluted and second in diluted form. The table shows that

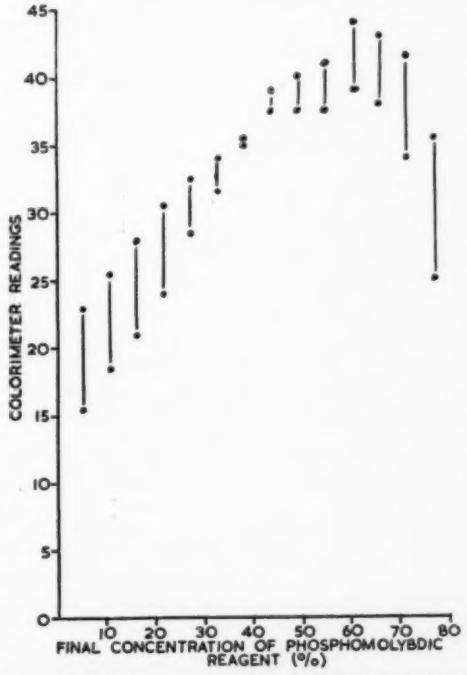

FIG. 4.—Addition of phosphomolybdic reagent made in diluted form. Reagent added as a 14-ml. quantity of a dilution in distilled water. At each concentration the upper point represents colour density at 4 minutes after addition of reagent and lower point density at 22 hours.

at 22 hours mixtures similar in final concentration of P.M.R. reached similar values in both experiments.

Discussion.—It is apparent that in the Folin and Wu method the density of colour produced is dependent not only upon the concentration of reduced copper but also upon that of the P.M.R. The fading which follows dilution with distilled water is due to change in the concentration of P.M.R.

Although the addition of reagent to reduced copper results in an immediate production of colour, any change of concentration of P.M.R. is followed by a slow change in density of colour until a new equilibrium is reached. The rapidity of this change varies directly as the difference between old and new densities.

Further experiments revealed a tendency for very high concentration of P.M.R. to retard the original production of colour. The slight fading which occurs with constant concentration of P.M.R. seems influenced by the absolute concentration of the reagent. It is likely that this is related to slight changes in the acidity of the final solution because increasing quantities of acid P.M.R. are added to a fixed quantity of alkaline copper solution. This fading can be largely inhibited by adjustment of acidity (Fig. 5).

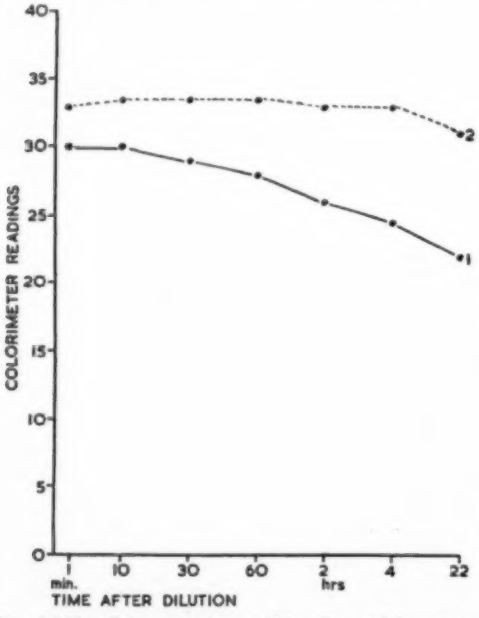

FIG. 5.—The effect upon colour stability of a small increase in phosphoric acid concentration in a dilute phosphomolybdic reagent. Change in colour density following the addition of 14 ml. of 16.5% reagent (1) compared with that found with use of a similar reagent in which the phosphoric acid concentration has been raised by 2.5% (2).

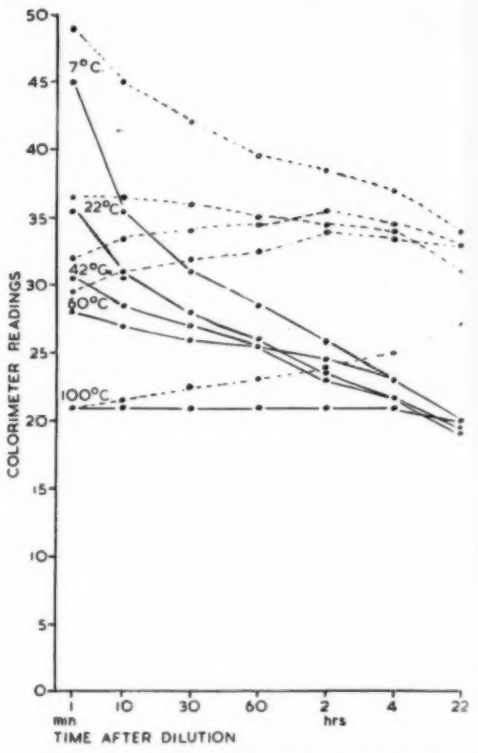

FIG. 6.—The effect of temperature upon colour production and stability. Reduced copper-phosphomolybdic reaction initiated and maintained for two minutes at five different temperatures before dilution with distilled water (continuous lines) and with ½ strength reagent (intermittent lines).

Effect of Temperature upon Colour Production and Stability

Several of the more successful modifications introduced to prevent fading involve raising the temperature of the reduced copper-P.M.R. reaction. The effect of temperature on this reaction was determined over a full range.

Technique.—The large Folin and Wu tube was employed in the manner already described, but after the six minutes' boiling the reduced copper was brought immediately to the temperature required and 10 ml. of P.M.R., already warmed or cooled, was added. The temperature was maintained for two

minutes and, after thorough mixing, dilutions made with distilled water and ¼ strength P.M.R., both at room temperature, and colour densities followed.

Fig. 6 illustrates the results. Initial colour production varied inversely as the temperature. Intensity of colour at 7° C. was more than twice that at 100° C. Those reduced copper-P.M.R. mixtures diluted with distilled water all arrived at similar densities at 22 hours; those diluted with ¼ strength P.M.R. tended to do the same.

Further dilutions with $\frac{1}{6}$, $\frac{1}{2}$, and $\frac{2}{3}$ strength P.M.R. were made with the mixture maintained for two minutes at 100° C., and Fig. 7 illustrates the results. All dilutions, except that with distilled water, increased in density of colour; the increase varied directly as the concentration of P.M.R. One-third strength P.M.R. and the undiluted sample showed similar curves.

Discussion.—In the Folin and Wu method temperature has a profound influence upon colour production and stability. The density of colour produced varies inversely as the temperature at which the reaction takes place. Any subsequent change in temperature results in a gradual change in density of colour to reach a new equilibrium.

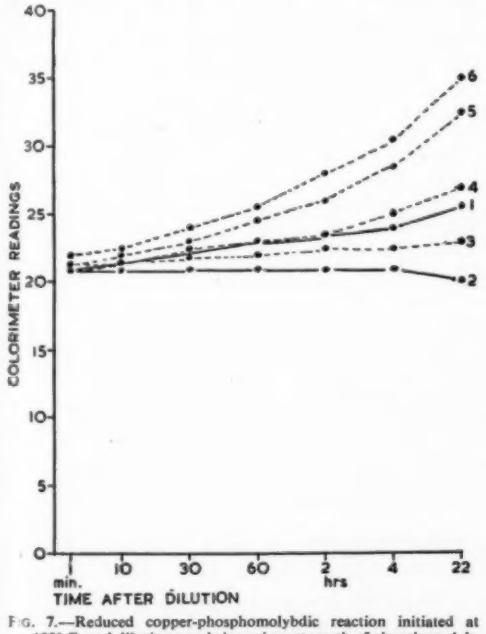

FIG. 7.—Reduced copper-phosphomolybdic reaction initiated at 100° C. and dilutions made in various strength of phosphomolybdic reagent. Change in density of undiluted mixture (1), diluted with distilled water (2), diluted with ⅛ strength (3), ¼ strength (4), ½ strength (5), ⅔ strength (6) phosphomolybdic reagent.

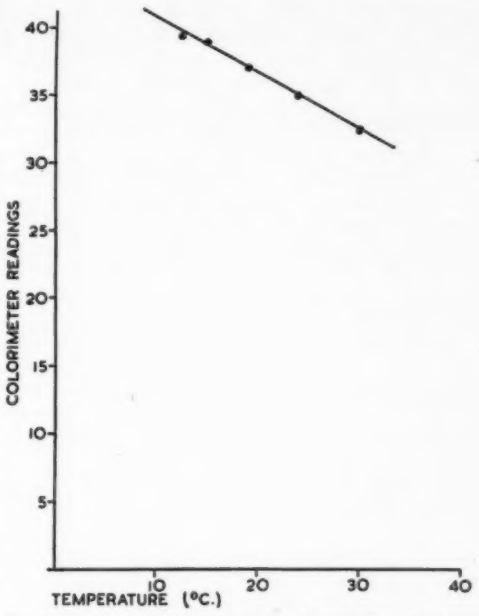

FIG. 8.—Colour density and environmental temperature. Variation in colour density encountered with a single standard glucose solution when the reduced copper-phosphomolybdic reaction is performed at different temperatures.

In these experiments the effect of temperature upon colour stability has to be distinguished from that of change in concentration of P.M.R. Also, the slight fading which occurs independently of these factors must be considered. The curve given by the reduced copper-P.M.R. mixture initiated at 7° C. and diluted with distilled water represents all factors favouring fading. The stability of colour in that mixture initiated at 100° C. and diluted with distilled water results from opposition of the two main factors.

When employing the classic Folin and Wu technique, the density of colour produced by a standard solution can be expected to vary from day to day, and from season to season with the environmental temperature. Fig. 8 illustrates the range to be expected.

Reduced copper solutions should not be cooled to below room temperatures by using running tap water (which in our laboratory is 10° C. below room temperature). The initial temperature of the reduced copper-P.M.R. mixture is largely that of the reduced copper, and so after dilution the initial colour fades at an even faster rate, for fading due to a rise of room temperature is added to that due

to a change in P.M.R. concentration. Also, with such supercooling, it is difficult to be certain that test and standard solutions are at exactly the same temperature when P.M.R. is added.

Photosensitivity of Reduced Copper-P.M.R. Mixture

Using the Folin and Wu method considerable variation in result was encountered when identical experiments were performed at different times of day. These variations were found to be due to a non-specific increase in density of colour which occurs on exposures of the reduced copper-P.M.R. mixture to sunlight.

Technique.—Standard glucose solution and a distilled water blank were treated with alkaline copper reagent using the classic Folin and Wu technique, but the P.M.R. was added in diluted form. A 14 ml. quantity was employed, consisting of 3 ml. P.M.R., 2 ml. of 25% phosphoric acid, 9 ml. distilled water. Each of the final solutions was dispensed into a pair of colorimeter tubes. One of each pair was placed close to a window on a sunny day but shielded from direct sunlight. The remaining tubes were kept in the shade. Colour densities were determined at four, 10, 30, and 60 minutes after addition of P.M.R. Tubes were in position at six minutes.

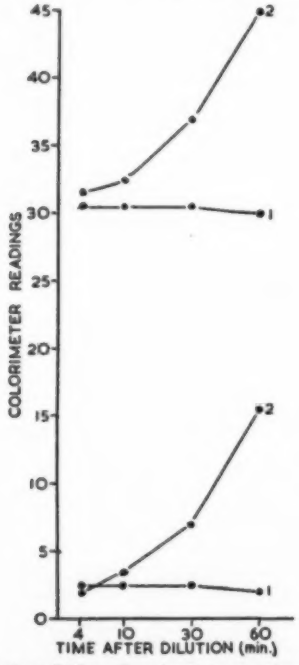

FIG. 9.—Photosensitivity of reduced copper-phosphomolybdic reagent mixture. Identical pairs of standard (upper) and blank (lower) solutions prepared. One of each pair left in the shade (1), the others (2) placed close to a window on a sunny day but shielded from direct sunlight. Tubes in position at six minutes.

The results are illustrated in Fig. 9. In spite of shielding from direct sunlight the increase in density was marked. By 60 minutes the blank had risen from 2 to 15¼ units and test 31½ to 45, i.e., each had risen 13¼ units. This increase is therefore unrelated to the reduced copper present. Exposure of the P.M.R. or copper reagent to light before mixing does not produce this colour increase when they are finally brought together. Boiling the alkaline copper reagent has no influence upon the production of this photosensitivity, for simple addition of P.M.R. to alkaline copper reagent results in a mixture of similar sensitivity.

It is felt that this phenomenon must explain the increase in density of colour of blank solutions previously observed (King and Garner, 1947) with alkaline copper and phosphomolybdic reagents. Blank solutions have been kept up to three days without any increased density of colour provided that they had been shielded from sunlight. A false impression of colour stability may be presented when a solution which is fading is exposed to sunlight. In practice care must be taken to avoid sunlight causing an increased density of colour. The danger lies in differential exposure of standard and test solutions.

Under normal laboratory conditions this presents no problem and provided the staff is aware of the danger no errors will arise. This applies to the Folin and Wu method, to all its modifications, and to methods employing similar reagents, such as those of Benedict (1931) and Haslewood and Strookman (1939).

Purity of Sodium Carbonate and Colour Stability

Previous workers (King and Garner, 1947) have stressed the importance of using absolutely pure samples of sodium carbonate in the preparation of alkaline copper reagents in order to prevent fading. Impure samples of carbonate have been used without any increase in fading being observed. A greater reduction was noted, but this was considered to be due to the bicarbonate content and the lowered alkalinity which increases the power of reduction of glucose (Harding and Downs, 1933). Blank colours were not increased when freshly prepared reagents were used.

Quantity of Reduced Copper and Colour Production and Stability

In all the experiments so far described a single standard solution containing 2.5 mg. of glucose

per 100 ml. was used. Experiments repeated, using stronger and weaker solutions, showed that alteration in colour density was always proportional to the initial colour developed. Folin and Wu and "true sugar" blood filtrates showed similar results.

Using the dilute P.M.R. in the manner already described, the standard curve shown in Fig. 10 was produced. The relationship between glucose and colour density was linear between 0.6 and 4.4 mg. glucose per 100 ml.; this is equivalent to

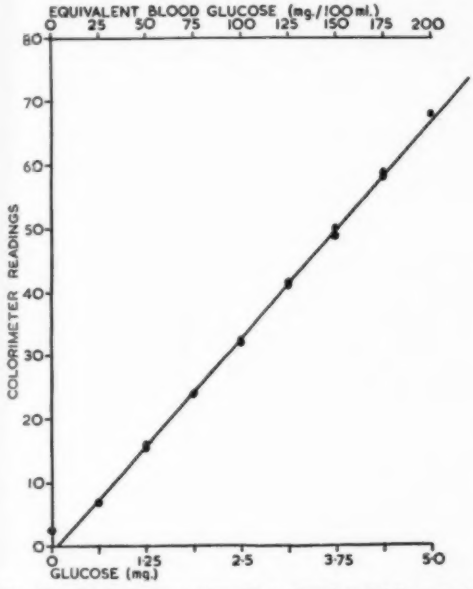
EQUIVALENT BLOOD GLUCOSE (mg./100 ml.)
0 25 50 75 100 125 150 175 200
COLORIMETER READINGS
0 10 20 30 40 50 60 70 80
GLUCOSE (mg.)
0 1·25 2·5 3·75 5·0

FIG. 10.—Standard curve using dilute phosphomolybdic reagent. The blood glucose equivalent assumes a dilution of 1 in 40.

25–175 mg. of blood glucose when, in the commonly used micromethod, blood is diluted 1 in 40.

It is apparent from the graph that a line drawn through zero and the reading of the 100 mg. standard faithfully represents the relationship between glucose and colour density in the range 25 to 175 mg. Above 175 mg. density increases out of proportion to glucose concentration.

Modifications of the Folin and Wu Method

Folin (1926 and 1929), using an increased amount of a modified P.M.R., suggests dilution with 1 in 5 strength P.M.R. to prevent fading.

This technique is largely effective in maintaining a stable colour. Using dilute reagent in place of distilled water the change in concentration of P.M.R. is limited. This feature has been adapted for use with standard P.M.R. (War Department Technical Manual, 1946), 1 in 5 strength P.M.R. being advocated as diluent. In our hands this still leaves appreciable fading; 1 in 3 strength is more effective (Fig. 2). The modified reagent, compared with the standard one, contains approximately twice the concentration of the effective constituents. By using the equivalent of a fourfold increase in P.M.R. concentration, the factors employed by the method of Sobel (infra) also help to maintain the colour stable. This technique has not been used widely because of difficulty in preparing the reagent, and because initial colour production is slow and time must be allowed for its full development.

Fiorentino and Giannettasio (1940) heat the reduced copper-P.M.R. mixture for five minutes in a boiling water-bath before dilution and reading.

Thomson (1946) adds P.M.R. to reduced copper immediately after boiling, removing tubes one by one at 15-second intervals and adding P.M.R. to each individually.

Fister (1950) adds sodium sulphate to the alkaline copper reagent, follows the Folin (1926 and 1929) technique, and boils for five minutes after P.M.R. is added.

Tonks (1952) adds P.M.R. to the reduced copper, which is not cooled, and boils for two minutes before diluting with distilled water.

The principal feature of these techniques is a raising of the temperature of the reduced copper-P.M.R. mixture and they are successful in producing stable colours. The lowermost curves of Figs. 6 and 7 represent these modifications. The result is achieved by balancing increased density due to cooling against the decrease caused by dilution with distilled water. When the reduced copper-P.M.R. mixture is diluted with distilled water the new density is almost exactly that which can be maintained at the new temperature and P.M.R. concentration. It should be noted that if the dilution is less than 1 in 3 a slight increase in density can be expected, and, if greater, then a fading will be observed. Of these methods only those of Fiorentino and Giannettasio and of Tonks are practicable. They can be criticized on two points. First, an essential feature of the Folin and Wu method is the accurate timing of the six minutes' heating in the boiling water-bath. This period must be exactly the same for both standard and test samples. Tonks does not cool immediately at the end of the boiling period, and he emphasizes that this must not be done. The reduction of the copper reagent must continue while the tubes are

still hot until P.M.R. is added. If a dozen tubes are involved, then the addition of the reagent must take at least a minute, leading to a variable treatment of the tubes. Secondly these modifications involve an extra manipulation and therefore an extra opportunity for technical error.

Sahyun (1936) adds sodium sulphate to the alkaline copper reagent and allows five minutes for the reduced copper-P.M.R. reaction to take place.

The Leitz photometer handbook (as quoted by Tonks, 1952) increases to 10 minutes the time allowed for the reduced copper-P.M.R. reaction.

Tonks found the method of Sahyun to have little advantage over the traditional method. The Leitz method he found to be partially successful in retarding fading.

We have increased up to 40 minutes the time allowed for the reduced copper-P.M.R. reaction with no effect upon fading when finally diluted with distilled water and do not consider these methods to be of value.

Sobel (1947) uses five times the normal amount of P.M.R. We agree with Tonks in finding such a technique successful. By increasing the quantity of P.M.R. two factors are employed. The amount of P.M.R. is large—sufficient to bring the final concentration in the reduced copper-P.M.R. mixture to beyond the peak of colour density at 60%. Dilution, provided it is not great, will bring the P.M.R. concentration back to a level giving a similar colour density on the opposite side of the peak (Fig. 4). Also at high concentrations of P.M.R. the rate of initial colour production and subsequent change are slowed down, helping to keep density constant. The use of such concentrations of P.M.R. is uneconomical and inconvenient in practice.

Lehmann and Silk (1952) dilute with 11.55% w/v phosphoric acid. They also remark upon the effect of not diluting after the addition of P.M.R. and also upon keeping final solutions in iced water.

In our hands the concentration advised gives appreciable fading. The use of a concentration of phosphoric acid equivalent to that in the undiluted mixture does retard fading, but nevertheless allows a significant amount.

The mechanism seems in the nature of a change in quality of the P.M.R. associated with an increase of phosphoric acid concentration relative to the molybdic acid. Lehmann and Silk claim that by incorporating a large quantity of phosphoric acid in the P.M.R. a similar stability can be achieved, distilled water being again used as diluent. This would be in keeping with the explanation suggested above.

The stability of the undiluted mixture has already been discussed. Storing tubes in iced water will result in the fading of dilution being apposed by the increased density caused by lowering the temperature.

A New Modification

The investigation has led to a further modification of the Folin and Wu method. The P.M.R. is added in diluted and slightly acidified form. The working reagent is constituted—P.M.R. 150 ml., phosphoric acid (sp.g. 1.75) 25 ml., distilled water 325 ml. The old and new techniques are compared.

Folin and Wu	*Modified Folin and Wu*
2 ml.	2 ml.
1. Blood filtrate or standard +	alkaline copper reagent
Heat in a boiling water-bath for exactly 6 minutes	
2. Cool (2–3 minutes)	
3. Add 2 ml. of phosphomolybdic reagent. Allow 2 minutes for reaction to take place.	3. Add 10 ml. of dilute phosphomolybdic reagent. After one minute mix and read in a colorimeter using a red filter.
4. Dilute with distilled water to total volume 12.5 ml. Mix and read in a colorimeter using a red filter.	

This technique not only eliminates the problem of fading but also shortens and simplifies the original method, so reducing opportunity for errors of technique. The danger of slight but significant differences of temperature between standards and unknown solutions at the stage of adding P.M.R. is greatly reduced, for the temperature of the reaction is dominated by that of the P.M.R.

The modified method has been in routine use for six months and has proved most satisfactory. Details are added as an appendix.

Summary

The density of colour in the Folin and Wu method is directly proportional to the concentration of phosphomolybdic reagent (P.M.R.) as well as the concentration of reduced copper. It is also inversely proportional to the temperature of the mixture.

Initial colour production is immediate, but change in concentration of the phosphomolybdic

reagent or in the temperature of the mixture is followed by a slow change in density until equilibrium is reached.

The alkaline copper reagent and phosphomolybdic reagent form a photosensitive mixture. Exposure to daylight results in the production of a blue colour unrelated to the amount of reduced copper present.

Previous modifications introduced to improve colour stability are discussed and their mode of action considered. Although some are successful they add additional manipulations to the original technique.

A new modification to the original Folin and Wu method is described, which both obviates fading and simplifies the original technique.

I wish to thank Group Captain Stamm for his advice and guidance in the preparation of this paper, the Director General Medical Services for permission to publish, and Corporal Whyley for technical assistance.

APPENDIX

Routine Procedure for Blood Glucose Estimation

A "true glucose" (Haslewood and Strookman, 1939) method of protein precipitation is employed.

Reagents.—The following are required:

1. *Isotonic Sodium Sulphate–Copper Sulphate Solution* (prepared by mixing 320 ml. of 3% $Na_2SO_4.10H_2O$ with 30 ml. of 7% $CuSO_4 5H_2O$.

2. 10% *Sodium Tungstate Solution.*

3. *Alkaline Copper Reagent* (Folin and Wu, 1920). This can be kept as two solutions, one containing the copper sulphate and the other sodium carbonate and tartaric acid. The reagent is then prepared freshly before use.

4. *Dilute Phosphomolybdic Reagent.*—This contains 150 ml. of phosphomolybdic reagent (Folin and Wu, 1920) and 25 ml. of phosphoric acid (sp.g. 1.75) in 500 ml. of distilled water.

5. *Working Standard Glucose Solution,* 2 mg.% in saturated benzoic acid solution.

Method.—Blood, 0.1 ml., is placed in 4.6 ml. of isotonic sodium sulphate-copper sulphate solution, then 0.3 ml. of sodium tungstate is added, thoroughly mixed and centrifuged.

Supernatant fluid, 2 ml., is pipetted into a Folin and Wu tube, and 2 ml. of the standard glucose solution into a similar tube. Then 2 ml. of alkaline copper reagent is added to each, mixed, and stoppered with non-absorbent cotton wool. The mixtures are heated in a boiling water bath for exactly six minutes and rapidly cooled to room temperature. Two minutes are allowed for the tubes finally to reach this temperature, then 10 ml. of dilute phosphomolybdic reagent is added to each.

The solutions are mixed by two inversions and read in a colorimeter, using a red filter.

Calculation.—$\left(\frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 100\right)$ mg./100 ml.

If the unknown is too deep to compare with the standard (reading above the equivalent of 180 mg. glucose per 100 ml.) dilute 1:1 with dilute phosphomolybdic reagent and multiply the answer by 2.

A distilled water blank should be included once a day to check the condition of the copper reagent. Blank values are not subtracted from standard or test readings. Blank solutions are not used for zero setting of the colorimeter.

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J. clin. Path. (1955), **8**, 262.

TECHNICAL METHODS

THE PRODUCTION AND STORAGE OF CONDUCTIVITY WATER

BY

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From the Central Middlesex Hospital, London

(RECEIVED FOR PUBLICATION OCTOBER 10, 1954)

Ordinary distilled water is not suitable for use in the estimation of lead, or for the production of colloidal gold solutions, and occasionally an ordinary still produces water of such impurity that it is unsuitable even for flame photometry. In such cases, glass-distilled water is used, but all-glass stills are expensive, fragile, wasteful of heat, and often bulky. We have found that we can obtain up to 20 litres a day of high-grade conductivity water suitable for the preparation of colloidal gold solutions or the estimation of lead by passing ordinary distilled water through a column of mixed-bed deionizing resin ; the apparatus employed is no more expensive than a glass still, mechanically strong, very economical in running costs, and requires only about 4 sq. ft. of bench space. If 6,000 litres of conductivity water are prepared a year from the poorest quality of distilled water the resin need be replaced not more than four times a year at a maximum cost of £1.

The design is such that water which has passed through the resin column comes into contact only with plastics which can yield no ions into solution: the deionizing column, storage container, and tap are made of polyethylene, the flexible tubing of P.V.C., and the wads of nylon mesh. The purity of the effluent is indicated by the rate of flashing of a neon signal tube.

Principles

Metallic ions are removed from solution by cation exchange resins, acidic ions by anion exchange resins. Water can be purified by successive passage through separate columns of the two types of resin, but a purer effluent is obtained from a single column containing a mixture of the two resins ; such a mixture is available commercially as " biodeminrolit " (Permutit Co.) or " amberlite monobed resin " (Rohm and Haas). We have used " amberlite M.B.1 " ; of this 1 ml. will remove about 0.6 mEq. of a salt, so that a column containing about 100 ml. resin will remove some 60 mEq. If the crude input contains 0.02 mEq/

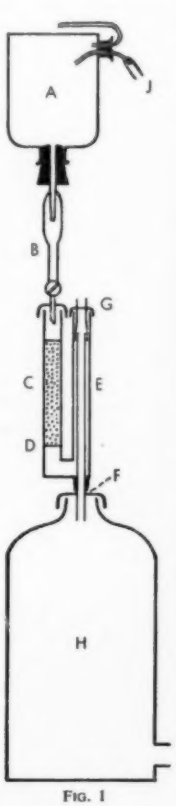
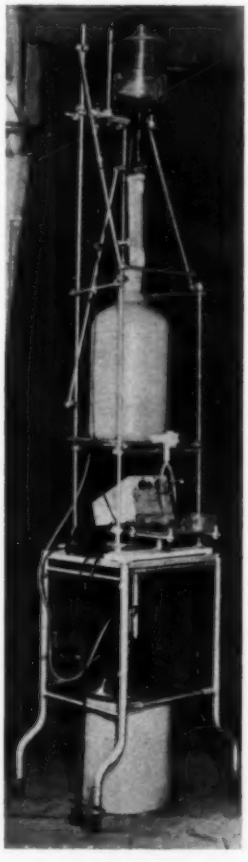


FIG. 1



litre (a very poor grade of distilled water), such a column will purify 3,000 litres before it is exhausted; when the resins are exhausted they can be regenerated should it be considered desirable.

Construction

The construction can be followed most easily by reference to Fig. 1.

A is a 5-litre aspirator bottle fitted with a drop sight-feed and tap B; ordinary distilled water is pumped up from a floor-level reservoir through the tube J using a "dymax" electrically driven pump to compress air into the reservoir. CDEF is the deionizing column made from ¼ in. wall polythene tubing of 1 in. lumen, formed into a U tube to ensure that the water level is always above the resin lest channelling occur with subsequent incomplete contact between resin and water. D is a polyethylene diaphragm occluding the lumen of the U tube, but perforated with 15 to 30 holes each about ½–1 mm. diameter. Above and below the resin are wads made of fine nylon mesh stockings, decolorized by treatment with stannous chloride-hydrochloric acid and sodium hydrosulphite-sodium hydroxide; they prevent the resin C from leaking through the diaphragm and from disturbance by the inflow.

E is the second limb of the U tube and F a 1-cm. tube sealed through the bottom of the U tube; the effluent flows through this tube into the storage bottle H, thus ensuring that the resin is always under water.

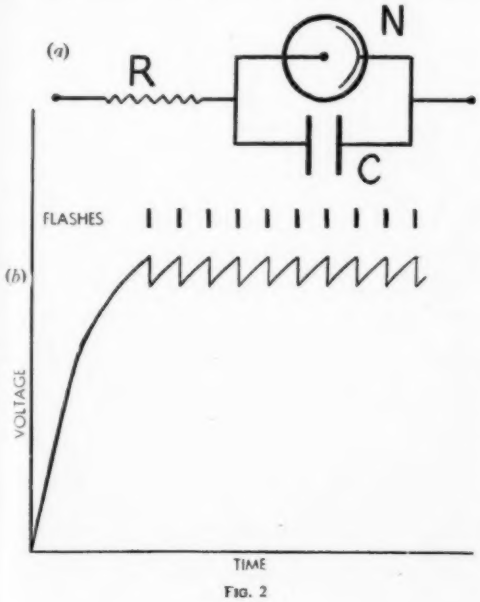


FIG. 2

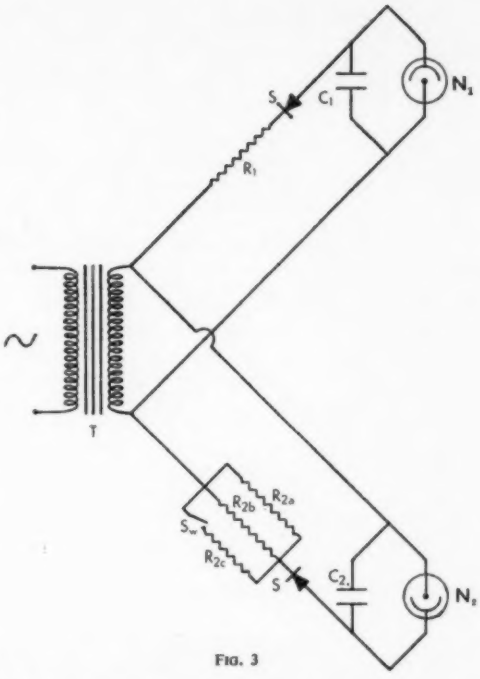


FIG. 3

G is a cap carrying two electrodes used for the neon tube indicator.

Polyethylene softens at a temperature of 150 to 200° C. and can be worked with a nitrogen torch. In this case, however, the U tube was constructed from four pieces of polyethylene tubing and the diaphragm D, which were welded together with a hot, thin, flat brass bit; the joints between the horizontal and vertical limbs were cut at an angle of 45°. The tube F is of polyvinyl chloride and was pushed through a polyethylene sleeve welded around a hole in the horizontal limb, and so tightly fixed therein that no leakage occurred.

Conductivity Indicator

This is a resistance tester based on the design of Knowles, Briggs, and Scragg (1951). The resistance in the circuit is estimated by the frequency with which a neon tube flashes; the higher the frequency the lower the resistance. A D.C. potential is set up across the ends of a circuit such as that of Fig. 2a, when the condenser C slowly charges through the resistance R. When the potential across the condenser reaches the striking potential of the neon lamp N, the lamp strikes and becomes a low resistance through which the condenser discharges. The lamp then ceases to glow and its resistance again becomes very large and the cycle starts again (Fig. 2b).

In practice the striking and cut-off potential differ by only about 5 volts. The platinum electrodes G (Fig. 1), of 0.7 cm.^{2} area and 0.5 cm. apart, formed part of R_2 in Fig. 3, which is developed from Fig. 2a.

T is a transformer giving 250 volts on the secondary: S are selenium rectifiers, 300 v., 60 mA; C_1 and C_2 are first-quality paper dielectric 4 m.f.d. 500-v. working condensers with an insulation resistance of several megohms. N_1 and N_2 are 5-watt indicator neons from which the ballast resistance has been removed; they strike at about 135 volts and quench at about 130 volts. R_1 is a fixed resistance such that N_1 flashes with a frequency of 20 to 60 per minute, and is of the order 150,000 to 200,000 ohms.

The resistance R_2 is composed of two parts in parallel: a fixed resistor R_{2a} larger than R_1 and the resistance R_{2b} between the electrodes G in Fig. 1. R_{2a} is chosen so that the frequency of the neon N_2 is considerably less than that of N_1 (about 400,000 ohms), and the electrodes G are such that a mixture of conductivity and distilled water in the ratio 9:1 will give a frequency somewhat greater than that of N_1. Thus, when N_2 flashes as fast as N_1 it is time to replace the resin. In practice, the frequency of N_2 is so low that the charge on C_2 leaks away before N_2 discharges, so that a testing switch Sw, which reduces R_2 to about 150,000 ohms and increases the rate of flashing to about 60 per minute, is fitted. R_{2b}, the testing resistance, is about 200,000 ohms.

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A METHOD OF SECTIONING ASPIRATED BONE-MARROW

BY

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From the Department of Pathology (Haematology), Postgraduate Medical School, London

(RECEIVED FOR PUBLICATION DECEMBER 3, 1954)

A technique for preparing histological sections of aspirated bone-marrow to be satisfactory should fulfil the following conditions: it should be simple and easy to perform; it should give a good concentration of marrow particles without much admixture with peripheral blood; it should produce very little lysis of the blood cells; and it should give good fixation of the tissue so that sections can be stained by Romanowsky dyes as well as by routine stains and nuclear details can be easily discerned. The technique described below is believed to satisfy these conditions and to have advantages over various methods so far described.

Other Authors' Methods

Mertens (1945) collected the marrow in a dry syringe and fixed the resulting coagulum in Zenker's solution. The sections contained marrow particles but also a large amount of peripheral blood.

White, Baker, and Griffin (1946) fixed the aspirated material in a mixture of nine parts of absolute methanol and one part of neutral formalin (40%). This method, although giving excellent fixation, failed to concentrate the fragments and also lysed the red cells.

Attempts at exclusion of peripheral blood were made by Schleicher (1947), Berman and Axelrod (1947), and Cappell, Hutchison, and Harvey Smith (1947). Schleicher's method consisted in picking up the gross fragments after illuminating the marrow aspirate from below, the smaller fragments being visualized after dilution with the fixative. This method is tedious and time consuming.

Berman and Axelrod (1947) gathered the fragments on a glass slide in a drop of topical thrombin solution, to which heparinized plasma was added, and the resulting coagulum was fixed. It was possible to rid the fragments of a large amount of the contaminating blood, but the concentration was not adequate.

These two methods were improved on by Cappell and his colleagues (1947) and by Hutchison (1953), who collected the marrow in large volumes of fixative and washed away the excess blood with larger volumes of water after fixation. The former used Zenker's solution minus acetic acid as fixative; the latter used a mixture of sodium sulphate, glacial acetic acid, formalin, and water. These methods, although they are elaborate and time consuming, achieved a good concentration of marrow particles. Lubitz, Greenwalt, and Dessel (1952) and Moomaw and Alt (1954) collected the marrow aspirate on unglazed filter paper and fixed the resulting clot containing the marrow

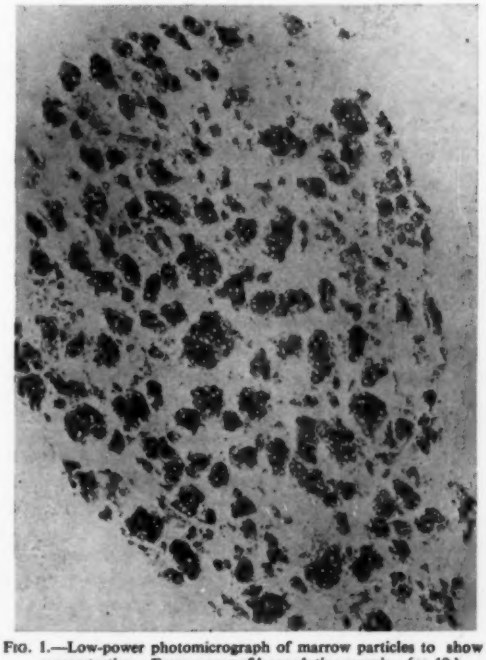

FIG. 1.—Low-power photomicrograph of marrow particles to show concentration. From a case of haemolytic anaemia. (× 19.)

fragments in Zenker's solution. However, in neither method was it possible to exclude blood from the marrow particles nor could a good concentration be obtained.

Method

The method now described gives a good concentration without much admixture with blood, and produces very little lysis of the red cells (Fig. 1). Fixation of the tissue and nuclear and cytoplasmic staining are satisfactory (Fig. 2).

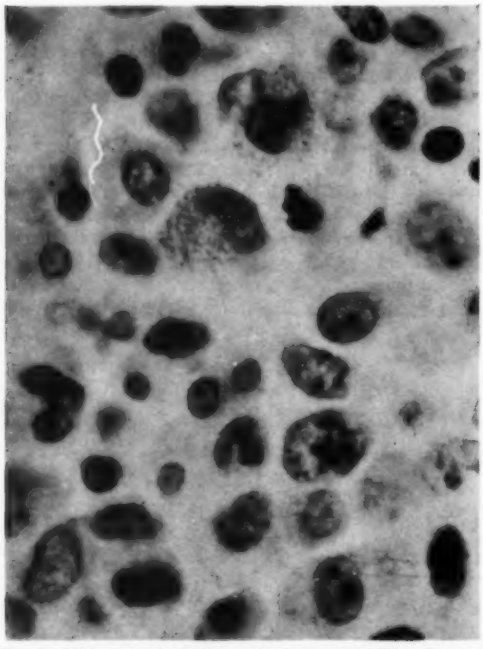

FIG. 2.—High-power photomicrograph of marrow particles. From a case of pernicious anaemia. (× 1,150.)

Fixative.—This consists of a mixture of equal parts of 15% formalin in saline and absolute ethanol. This solution has a specific gravity of about 0.930, i.e., almost exactly that of human fat—0.92 to 0.94 (Behnke, 1945). When the marrow aspirate is added to the fixative the blood remains in suspension and the coarse marrow fragments rapidly settle down to the bottom of the container; even fatty marrow settles down in a few seconds.

Procedure.—Marrow aspirate, 0.25 ml., is added to 20 ml. of the fixative in a screw-capped bottle and thoroughly mixed. It is then allowed to fix overnight.

In the morning the sediment is resuspended by gentle shaking and the coarser fragments are picked up with a glass pipette after they have settled to the bottom of the bottle, which usually takes only a few seconds. They are then transferred to a centrifuge tube containing 70% ethanol, which is then closed with a rubber bung. The material is subsequently dehydrated with two changes of absolute ethanol for one hour each. The alcohol is then drained off and replaced with a 2.5% solution of "necol"* in equal parts of absolute ethanol and ether and left overnight. This preliminary embedding in "necol" is necessary to hold the fragments together before they are finally embedded in paraffin wax (White *et al.*, 1946).

On the third day the excess "necol" is drained off and replaced with 2–3 ml. of chloroform to harden the collodion. After 30 seconds the aggregated mass of fragments is displaced by gently warming the tip of the tube over a very weak flame. The mass of fragments is then cleared with two changes of benzene and embedded in paraffin wax.

Summary

A simple method is described for preparing histological sections of aspirated marrow. It gives adequate concentration of marrow fragments with very little lysis of red cells and good fixation of tissue.

This work was undertaken during the tenure of a Nuffield Foundation Fellowship.

I have to thank Dr. J. V. Dacie and Dr. D. D. L. Mollin for their guidance. The histological sections were cut and stained under the direction of Mr. J. G. Griffin; the photomicrographs were taken by Mr. C. A. P. Graham.

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* Necol is obtainable from B.D.H. and sold as Necol Collodion Solution 301–261 (356/A9).

J. clin. Path. (1955), **8**, 267.

BREWER'S MEDIUM AS A METHOD OF INDUCING SICKLING IN SUSCEPTIBLE CELLS

BY

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From the Pathology Department, Marston Green Maternity Hospital, near Birmingham

(RECEIVED FOR PUBLICATION SEPTEMBER 18, 1954)

While investigating possible causes of anaemia, cholaemia, and uraemia in a woman of pure Negro stock, sickling of the erythrocytes was found in wet-blood smears. (Electrophoretic investigations of the patient's haemoglobin demonstrated that the patient was a heterozygote, showing the sickle-trait pattern A–S.)

The original investigations were undertaken during a haemolytic crisis, and among the investigations a blood culture was made using glucose broth and Brewer's medium. The media remained sterile until discarded after 10 days. It was found that the glucose broth contained blood of normal appearance, apart from slight crenation of the red cells.

On removal of a sample of blood from the Brewer's medium it was seen that the red cells were sickled, and that no attempt was made by the cells to revert to normal contours. The Brewer's medium had been sealed by a cotton-wool plug inserted in the mouth of the tube, but no oil had been placed on the surface of the medium. The experiment was repeated and several smears made and stained by Leishman's method. The cells were typical of sickle-cell disease, and resembled those found by other methods, e.g., vaseline-sealed wet smears, blood-under-oil in Wintrobe oxalate mixture, in heparinized tubes, and in sealed wet smears containing *Bact. coli*. With all these methods formalin has to be used to fix the cells in the sickled state. This gives adequate fixation, but the subsequent staining is not always satisfactory.

The use of Brewer's medium not only obviates the necessity of using formalin to fix the red cells in the sickled state, but provides material from which smears can be stained in the ordinary routine manner.

Brewer's medium is nutrient broth containing 0.1% of sodium thioglycollate (thiolacetate) and 0.05% of agar. The induction of sickling and the subsequent fixation of the sickled cells would appear to be due to the thioglycollate present.

We are grateful to Dr. Hermann Lehmann, of the Department of Chemical Pathology, Saint Bartholomew's Hospital, London, for his report on the electrophoretic pattern of the patient's haemoglobin.

J. clin. Path. (1955), **8**, 268.

OBITUARY

PROFESSOR HEŘMAN ŠIKL

1888–1955

Professor Heřman Šikl, who died in Prague at the age of 66 on January 16 of this year, was a member of the General Advisory Board of this Journal.

Professor Šikl studied medicine at the Charles IV University, Prague. After graduation, in 1913, he worked under Professor Thomayer and studied pathology under Professor Hlava, the founder of the Czech school of morbid anatomy. In 1921 he was appointed *Privatdozent* and in 1928 assistant professor. In 1937 he became full professor and director of the Hlava Institute of Pathology at the Charles University. In this post he remained until his death. In the course of his tenure of this post, Professor Šikl built up the Czechoslovak Bioptic Centre for the diagnosis of tumours, which covers the whole of Czechoslovakia. Now, over 20,000 specimens from all parts of the country are being examined and reported upon in this centre yearly.

Professor Šikl regarded pathology as the basis of clinical medicine: throughout his career he maintained the closest touch with his clinical colleagues; the full facilities of his department were always at their service and all his researches were aimed at throwing light upon problems of clinical medicine. Very early in his career he described the nature of the exanthem of typhus and later was even able to identify rickettsiae in the skin rash. He was one of the first to demonstrate the presence of protein crystals in the kidney in diffuse plasmocytosis; he did much work on cardiac disorders and believed that he had proof of the allergic nature of certain types of myocarditis. Professor Šikl's technique of dissection of the heart by transverse sections is largely applied, particularly on the Continent, in the study of cardiac malformations. He was one of the first to draw attention to, and to study, the carcinoma of the lung prevalent in the workers in the uranium mines of Jáchymov.

Professor Šikl was a lucid, stimulating, and enthusiastic teacher; he regarded his lectures as constituting the most important aspect of his professorial duties, many and arduous though they were. Throughout a protracted period of ill health, and almost up to the end of his life, he remained assiduous in his role as a teacher. Both as teacher and director of studies, he inspired the regard and affection of all who worked under or with him; as examiner he inspired esteem and confidence. He was a fluent linguist, equally at home in his own language, English, and German. His contributions to scientific periodicals, numbering over 100, were made in all these languages with equal ease and grace. He was an entertaining and witty conversationalist in all these languages. In his younger days he had been a skilled mountaineer, skier, and canoeist; throughout his life he retained a keen interest in literature and music. To the end he remained truly young in spirit. To those who knew him, his departure renders the world a duller place.

Professor Šikl was a member of many scientific societies, among others of the (British) Association of Clinical Pathologists, to which he was elected as corresponding member in 1948.

Professor Šikl married, in 1925, Milada Matejovská, of Prague, who survives him. There were no children of the marriage.

S. C. D.

BOOK REVIEWS

Ciba Foundation Symposium on Leukaemia Research. Edited by G. E. W. Wolstenholme and Margaret P. Cameron, assisted by Joan Etherington. (Pp. xiv+ 297; 56 figures, 30s.) London: J. & A. Churchill. 1954.

The principal papers in this book are not very different, except perhaps in their uniformly high quality, from those read at any pathological congress. The stimulating quality of the book, and it is very great, lies in the verbatim reports of the subsequent discussions. Leading workers on leukaemia, brought together *in colloquio* by the Ciba Foundation, freely expressed their minds not only on established knowledge and the facts newly presented to them, but also on what seemed to them the most likely course of future advance. The reader ends confirmed in his ignorance of the fundamental nature of leukaemia, impressed by the pace of recent advance in its chemotherapy, but above all refreshed by intercourse with minds that see further and wider than most of us can.

MARTIN HYNES.

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IMMUNOLOGICAL ANALYSIS OF ABNORMALITIES IN THE HUMAN SERUM PROTEINS BY A GEL-DIFFUSION METHOD

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Immunological methods allow of a more precise discrimination between similar proteins or high-molecular-weight carbohydrates than any chemical or physical method; but they have in practice been little used in analysis. There are a number of reasons for this: only substances which are antigenic can be identified; the major component of a mixture may provoke less antibody than a contaminant, or none at all; there is no simple way of correlating a precipitin reaction curve with the physical properties of the antigen molecule—shape, molecular weight, and so on; quantitative estimation is a laborious and specialized technique; furthermore there is no certainty that all the antibody measured has been brought down by the protein under investigation, rather than by contaminating antigen-antibody systems in the same optimal range.

The use of gel-diffusion methods such as those described below has altered this situation. It is now possible to identify a single line or "front" with near certainty as being due to a particular protein; and, used with suitable precautions, this method of identification is far more specific and sensitive than any purely physico-chemical technique. It avoids the laboriousness and the uncertainties of immunological estimation, though it sacrifices its quantitative precision. However, quantitative estimates are possible, of a grade of accuracy usually sufficient for practical purposes.

The present study illustrates some clinical applications of the gel-diffusion method, with an approach to the problem of quantitative estimation. The method used is based on those of Ouchterlony (1948, 1949), Elek (1949), and Grabar (1954), in which the reagents diffuse towards one another from cups cut in agar in petri dishes.

Under these circumstances the reagents will meet at "optimal proportions" somewhere in the intervening space and a band of precipitate will occur there. If several antigen-antibody systems are present, several bands may develop, each corresponding with one antigen. If two differing preparations of antigen are placed in cups near one another, and both diffuse towards the antiserum, the lines due to proteins present in both will join at an angle, while if a particular protein is present in one but not in the other its characteristic line of precipitate will not be diverted (Fig. 1).

The methods using diffusion in test-tubes, such as those of Oudin (1946), Munoz and Becker (1950), and Oakley and Fulthorpe (1953), though giving well-marked band-patterns, do not allow of this side-to-side comparison of different preparations. Clearly with the petri dish method any convenient number of antigens can be arranged round the central antiserum cup, so that the lines due to a common antigen may join to form a polygon; in practice, with fairly strong antisera, eight or 10 is the maximum giving lines of reasonable length.

A further refinement of technique is the combination of micro-electrophoresis (Grabar and Williams, 1953) with gel-diffusion. In this method the antigenic components are first separated by an electrophoretic run in agar, and then the antiserum is allowed to diffuse towards them from a trough cut at a suitable distance.

These techniques have as yet hardly been applied to the study of pathological sera. Clearly they are most readily adapted to showing qualitative or gross quantitative changes, their chief merit for this purpose being their very high specificity. Within their limits—that is, the power of discrimination of the antibody-forming cells of the animal species producing antibodies—there can be no sort of doubt about the identity of the fractions in which alterations occur. In the case of human sera, the antisera used in this work differentiate normally some dozen serum components, and other lines can be identified in

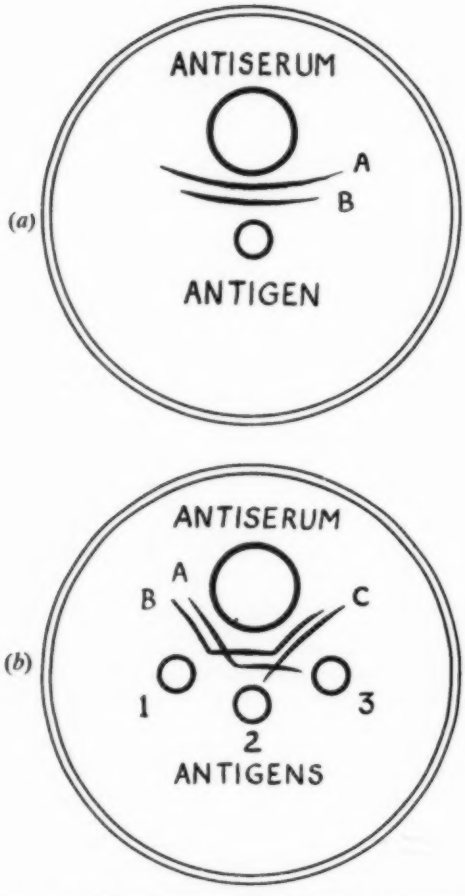


FIG. 1.—(*a*) Crude preparation of antigen (containing components A, B) diffuses towards antiserum where each pair meets as optimal proportions bands of precipitate form. (*b*) Three crude preparations of antigen diffuse towards antiserum—1 is seen to contain components A, B; 2 contains the same components but in different relative proportions since the lines join with those from 1; 3 contains one common component B and one fresh component C.

special cases by the use of fractionated or "pathological" sera (see below). Grabar and Williams (1953) quote at least 15–17 substances as being demonstrable. This very simple method for distinguishing so many different proteins can be used for analysis of pathological sera in large numbers, and may both throw light on the function of individual proteins and prove a more refined aid in diagnosis than paper electrophoresis alone. Useful results have been obtained in the study of a- and hypo-gammaglobulinaemia, and promising ones in nephrosis, multiple myeloma, etc.; some of these are described below.

Technique

Antisera.—These were raised in rabbits either by the use of alum-precipitated antigens given intravenously (Kabat and Mayer, 1948) or by subcutaneous injections of emulsions made according to the method of Freund and McDermott (1942). All sera are preserved with 0.02% sodium azide. The antigens used should in general be as crude as possible, that is, they should contain all the proteins which will later need identification; special investigations will of course call for partially purified antigens. Satisfactory antisera will contain of the order of 0.25 mg. precipitable nitrogen per ml. Individual rabbits are exceedingly diverse in the pattern of antibodies which they produce to a multiple antigen, even if the total weight of antibody varies little, and there is some advantage in not bulking antisera but in keeping them separate.

Medium.—For protein antigens, agar is the most convenient medium. In this work New Zealand agar (Davis) has been used throughout at 0.5–0.6%, made up in phosphate buffer saline (*p*H 7.4) containing sodium azide (0.02%); this is filtered and poured in specially selected flat-bottomed petri dishes to a depth of 3 mm., care being taken to keep the dishes perfectly level. (Agar may be unsuitable for systems containing carbohydrate antigens, for obvious reasons.)

Setting up the Reaction.—For satisfactory results it is necessary to keep the arrangement of the cups strictly uniform: the exact dimensions will depend upon the strength of the antiserum. With antisera of the antibody content quoted above, a distance of 10 to 12 mm. between the edges of the antiserum and antigen cups, and about the same distance between the individual antigens, is sufficient for clear differentiation of the lines. With these distances the results can be read at 18 hours; if even a slightly greater distance is allowed the time is sometimes considerably increased. Such dimensions allow a symmetrical arrangement of six to eight cups for antigens surrounding the antiserum cup.

It is convenient to cut the cups by means of corkborers; their size and distances apart can thus be varied at will, which is impossible if a template is used. It is not necessary to seal the bases of the cups. The nearer the reagents are together, the easier it is to get visible lines with weak or dilute antisera. At 4 mm. one drop of a strong antiserum diluted 1/32 gives a clear line (Fig. 2). The limiting dilution of antiserum is that which just gives a positive ring (interfacial) test.

Antigen Concentration.—For clear-cut lines it is necessary that the total amount of antigen in each cup should be approximately in "equivalent proportions" to the total amount of antiserum, but a fairly wide variation is permissible (Fig. 3). If there are two

antigen-antibody systems present whose optimal ratios are very widely different, it may only be possible to bring one of them out satisfactorily. It is desirable in starting on any new batch of antiserum to put up a

FIG. 2.—Limiting concentration of antibody. The cups 1–6 are filled with human serum albumin, decreasing by serial fourfold dilutions from 1% to 1/1024%, the cups 7–11 with antiserum at 1/1, 1/4, 1/16, 1/64, 1/256 (2 drops of each): the distance from antigen to antiserum is 4 mm. The limiting concentration of antibody under these circumstances is seen to be 2 drops of 1/64, assuming that the reagents are at approximately optimal proportions.

plate containing serial dilutions of standard antigen against a standard volume. With most of the systems used in this work, 4–24 drops of antiserum, 1–4 drops of antigen (human serum, undiluted) at 11 mm. gives a satisfactory general test of a pathological specimen. When the nature of the change is known the proportions can be altered, absorbed sera used, etc., to bring it out clearly and if possible quantitatively. Fig. 5 shows an example where the use of absorbed sera, and lesser distances, brings out with a very high degree of sensitivity the effects of therapy in a case of hypogammaglobulinaemia.

Identification of the Lines.—With human serum this is most conveniently done by means of fractions eluted from electrophoretic paper strips. The albumin and the γ-globulin can be easily identified with certainty in this way. The β-globulins form a "family" containing four members at least; this fraction is always contaminated with α_2-globulin, which with our standard (rabbit) antiserum has always appeared to be a single entity, though Grabar and Williams (1953) using horse antisera find several. The α_1-globulin has not been studied in this investigation. These identifications can be checked by means of a modification of the agar-electrophoresis method of Grabar and Williams (1953) as described below.

Quantitative Estimations.—The amount of antigen in a sample influences both the distance away that its line develops, and the density of the line. The distance is not at all sensitive to small alterations; in one system it was found that 2× changes in concentration had the effect of shifting the line one millimetre. However, it is often sufficient to know the order of concentration, particularly where one is investigating the deficiency of a protein—a research to which the method, because of its high sensitivity, is particularly applicable. Thus a side-by-side comparison of the pathological serum with normal serum at a known dilution or with a known concentration of γ-globulin (Fig. 4) gives a useful working estimate of the amount of γ-globulin present. More precise methods of estimation are described below. In the rare cases in which there is an absolute deficiency of a protein the method can be pushed to the limit of its sensitivity, and is far more sensitive than any other at present available (Fig. 6).

Recording and Preservation of Completed Reactions.—The photographs reproduced here were obtained by placing the petri dish on the horizontal lens of a Leitz projector and photographing the image on the screen; this is only practicable, however, with this type of projector. Attempts to photograph the reactions on a black background by obliquely transmitted light were only moderately successful.

It is quite easy to make the preparation permanent by fixing with formalin, washing thoroughly, removing

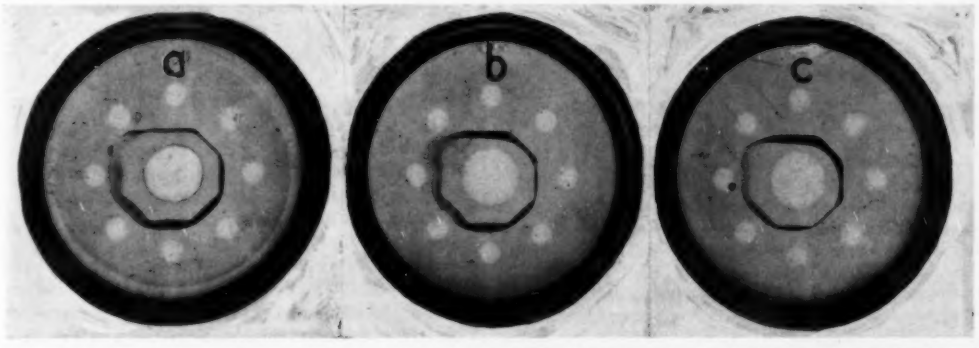
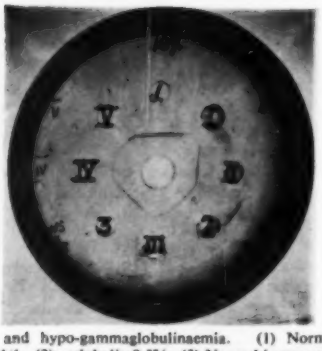


FIG. 3.—Optimal proportions. Three different amounts of antiserum (*a*, 16 drops, *b*, 8 drops, *c*, 4 drops) against eight serial dilutions of antigen (human serum albumin: 4 drops of 1%–1/128% arranged clockwise starting at the top). The optimum (sharpest line) is clearly 1/2 : 16 drops, 1/4 : 8 drops, 1/8 : 4 drops.



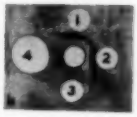


FIG 4.—A- and hypo-gammaglobulinaemia. (1) Normal human serum 1/1. (2) γ-globulin 0·3%. (3) Normal human serum 3/10. I, Patient G. P. (Grant and Wallace): "total a-gammaglobulinaemia." F., 23 yr. II, Patient P., F., 47 yr. III, Patient N., F., 33 yr. IV, Patient W. F., M., 3 yr. V, Patient H., F., 38 yr. The deficiency of γ-globulin in the samples of serum from patients II–V can be seen by the faintness of the line of precipitate and the decrease in its distance from the (antigen) source. The line from normal serum 1/1 is in "antigen excess" to the amount of antiserum present. There is no sign of the presence of any antigen in Serum I.

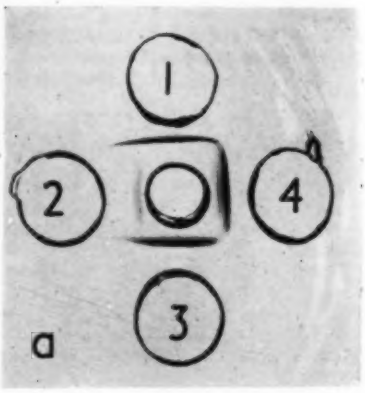


FIG. 6.—A-gammaglobulinaemia. These two reactions demonstrate a more rigid test on the serum of Case 1 for traces of γ-globulin. (a) 1, Normal serum 1/100. 2, Serum, Case 1. 3, Serum, Case 2. 4, Serum, Case 3. While Cases 2 and 3 show more γ-globulin than normal serum at 1/100 (their lines are further from the antigen source), there is still no sign of a junction with any line from the serum of Case 1 (the other lines visible are due to minor globulins). (b) 1, 2, normal serum at 1/1,000; 3, normal serum at 1/10,000; 4, serum, Case 1. Compared with (a) the antiserum has been diluted 1/10; distance is 2 mm. There is still no sign of any deviation of the γ-line, i.e., the amount of γ-globulin present in Case 1 serum is (if any) less than 1/1,000 of the normal, but, since normal serum at 1/10,000 is too weak to react at all, there may be the equivalent of this amount at least present.

the agar slab, and drying it on a lantern slide. When completely dry the fainter lines disappear: the plates may either be stored dry and moistened for subsequent re-examination, or covered before they are quite dry with several coats of "betacryl 122X" (I.C.I. Dyestuffs), a plastic soluble in xylene. The final coat of plastic might be oiled or varnished to render it impermeable to water.

Such preparations, if they are not too thick, give better results in a projector than a photographic lantern slide.

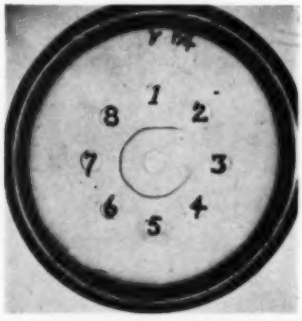


FIG. 5.—Effect of therapy on a case of hypogammaglobulinaemia. Case 4 (a boy of 3) had less than 0·05% gammaglobulin initially. He was given 0·5 g. of gammaglobulin weekly for four months, during which time the serum γ-globulin rose to about 0·25%. This figure illustrates this response to therapy. (1) γ-globulin (of the batch administered) 0·3%; (2) patient's serum before start of treatment; (3) one week; (4) three weeks; (5) five weeks; (6) nine weeks; (7) 13 weeks; (8) normal serum ¼. The antiserum has been "adsorbed" (with the serum of Case 1) and reacts only with γ-globulin.

Illustrative Cases

Hypogammaglobulinaemia.—Cases of this syndrome are being more frequently found with the increasing use of paper electrophoresis in clinical laboratories. Fig. 4 shows a routine test on the serum of five cases, in four of which γ-globulin is shown to be present though reduced in amount, while in one (Case 1) it appears to be absent entirely. This case has been described by Grant and Wallace (1954) (to whom my thanks are due for the supply of samples): at the highest antiserum dilution practicable no suggestion of a γ-globulin line was found.

Patients with hypogammaglobulinaemia usually present as recurrent pneumonia, bronchiectasis, mastoiditis, etc., the deficiency of γ-globulin being correlated with a deficiency in antibody defences. Of the cases from which serum samples have been examined here, four (Cases 1, 2, 3, 5) were in adult females, and one in a boy of 3 (Case 4). In all except Case 1 there is some indication from the history that the condition was not present at birth; in the child the first evidence of deficiency appeared at the age of 1, in Cases 2, 3, and 5 during the 20s or late 'teens. None of the cases had any signs of deficiency of the

lymphoid system, as far as could be ascertained by clinical examination and blood counts. A number of cases of this syndrome have recently been described in Europe and the U.S.A. (Bruton, 1952; Young and Wolfson, 1954; Janeway, Apt, and Gitlin, 1953), some with and some without changes in lymphoid tissue.

Treatment is directed towards replacement of the absent antibodies, either with plasma, or preferably, because of the lesser risk of jaundice, with purified γ-globulin. These materials usually contain a sufficient titre of antibodies against the commoner infections to afford a reasonable measure of protection. Janeway *et al.* (1953) state that a blood level of 0.1 to 0.15% γ-globulin is necessary to obtain therapeutic results. This is about the limit of sensitivity for paper electrophoresis, and quantitative results are hardly possible. Fig. 5 shows the effect of therapy and Fig. 7 an attempt at quantitative immunological estimation: in the original plate at least there was

FIG. 7.—Quantitative estimation of γ-globulin. "Reference" (1) γ-globulin, (2), (3), (4), (5), (6), (7), (8) volumes of serum from Case 4 on 14/3 which if matched with (1) give equivalent concentrations of 0·1, 0·15, 0·2, 0·25, 0·3, 0·35, 0·4 (approx.). The three reactions, 2, 3, 4, on the right are definitely in "antigen excess" (the first interferes with the precision of part of the "reference" line): careful comparison of the characteristics of the lines on the original plate showed that (6) gave the best match to the density and sharpness of (1). It has not been possible to bring this out convincingly in the photograph.

no doubt, from comparison of the density and diffuseness of the lines, that 0.25 to 0.30% γ-globulin was equivalent to the concentration in the serum under test. This sample was obtained from the child of 4 (Case 4), who was being treated with 0.5 g. of γ-globulin intramuscularly once a week; the sample was taken just before an injection, and, since it represented presumably a minimum concentration, the treatment had evidently satisfied the criterion of Janeway *et al.* quoted above.

Comparison of Serum and Urinary Proteins in Nephrotic Syndrome.—Another clinical condition in which the specificity of the immunological method is of value is in the examination of the urine in proteinuria. Fig. 8 shows the result of a parallel examination of urine and serum in two

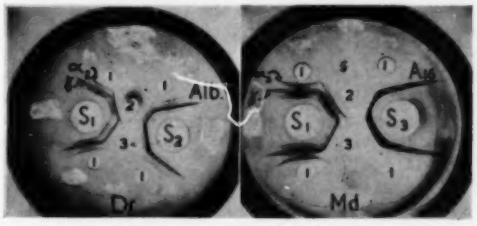


FIG. 8.—Nephrotic syndrome: serum and urine proteins. The serum and urine of two cases (Dr, Md) of nephrotic syndrome analysed with the assistance of three antisera: S_1 reacting with γ-globulin, α_2-globulin and some others (but not albumin), the others (S_2, S_3) reacting mainly with albumin. (1) Normal serum 1 vol. (2) Patient's serum 1 vol. (3) Patient's urine 2 vol. In both plates α_2-globulin appears to be absent from the urine: γ-globulin is present in both, and albumin is in very high concentration in the urines and deficient in the sera.

cases of the nephrotic syndrome. The urinary threshold of excretion closely parallels the molecular weights, α_2, and to a lesser extent γ, being retained in the serum, and albumin appearing in high concentration in the urine.

This figure also demonstrates the antigenic identity of the urinary and the serum proteins. As far at least as the rabbit antibody-producing system can discriminate, the process of excretion does not appear to have had any deleterious effect upon the protein molecules.

Serum Protein Abnormalities in Multiple Myelomatosis.—Fig. 9 shows the results obtained with myeloma sera and the antisera raised against them. Fig. 9a shows the results with a "γ-myeloma" with a typical electrophoretic picture: here the increased γ is evident in both the homologous and the heterologous (anti-normal-serum) reactions, i.e., the increase is due to excess of serologically normal γ-globulin, though whether the γ_1- or γ_2-fractions are involved is not shown by this method. In the β-myeloma the reaction with anti-normal-serum shows no marked deviation from the normal pattern, though the proportions of some of the components are changed, but the homologous reaction shows the presence of a strong antigenic component, apparently absent from normal serum, though further analysis suggests that the antibody produced cross-reacts with one of the normal β-globulins.

Finally Fig. 10 shows the reactions of a serum which contains a globulin of an unusual kind. This substance was precipitated on dilution of the serum

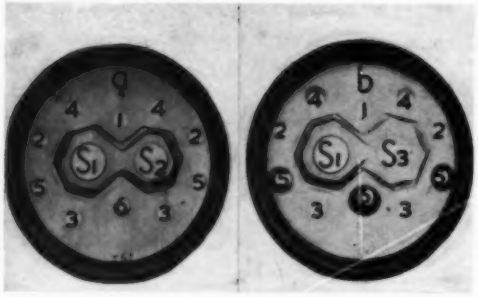


FIG. 9.—Multiple myeloma. (a) " Gamma myeloma " (Case Ou). 1, 2, 3 = Normal serum, 4, 5, 6 = myeloma (Ou) serum, at ¼, 1, and 2 drops respectively. S_1 = Antiserum raised against normal human serum, reacting mainly with γ-globulin (outer strong line) and albumin. S_3 = Antiserum raised against serum of myeloma patient (Ou): reactions similar. (b) Beta myeloma (Case Pk). Arrangement as in (a), but the serum used is from Case Pk and the antiserum S_3 was raised against this. The reactions of serum Pk with " anti-normal-serum " are much the same as those of serum Ou in A, but reaction with the homologous S_3 reveals the presence of an apparently " specific " component.

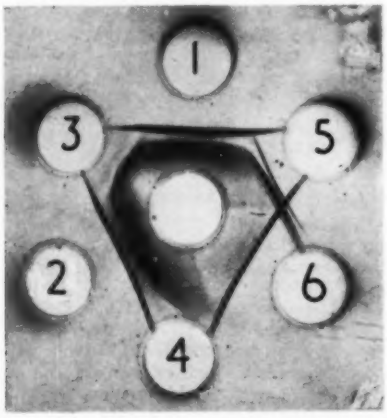


FIG. 10.—Abnormal globulin. 1, Normal serum. 2, Serum of patient Ed. 3, Fraction from serum of patient Ed. 4, Fraction similarly extracted from normal serum. 5, Fraction eluted from electrophoretic strip of serum Ed. 6, Serum of Case 1 (a-gammaglobulinaemia): the antiserum is against normal human serum. The reaction is put up at relative " antigen excess " for most serum components. The reactions with 2 and 3 do not show the presence of the abnormal protein owing to interference by the γ-globulin reaction (the fluffy innermost line). The well-marked outer line is due to α_2-globulin, removed by fractionation from 3, 4, and 5. The sharp outermost line opposite 5 is due to an abnormal protein, which has here been concentrated relative to γ-globulin and therefore brought out clearly. Note that it is " deviated " by normal serum (1), though not by the a-gammaglobulinaemia serum (6), hence it must be present in normal serum (though at too low a concentration for its faint line to appear on the photograph), a conclusion which is inevitable since antibodies to it have been provoked by the normal human serum used as the original antigen. But its metabolism is apparently linked in some way with γ-globulin, in spite of complete lack of serological cross-reaction, unless its absence from the a-gammaglobulinaemia serum is purely coincidental.

with three parts of water, and had haemagglutinating properties, although it did not appear to be an antibody in the strict sense. The patient suffered from haemolytic anaemia, and the " haemagglutinin " was assumed to have something to do with this. The interesting point brought out is that this very unusual substance is evidently present in normal serum, in very low concentration, and is there strongly antigenic; not only does the antiserum raised against normal serum contain an appreciable amount of antibody against it, but it can actually be observed as a hitherto unidentified faint line in the normal serum pattern, very near the antigen source, i.e., in low concentration, but definitely joining with the line produced by the purified substance from the pathological serum.

Combination of Immunological Analysis with Electrophoresis.—The method described by Grabar and Williams (1953), in which antigen is electrophoretically separated in agar before the reaction with antiserum, is the one of choice in certain cases, but it suffers from some limitations for routine use; though technically fairly simple there are more things which can go wrong than in the petri-dish method, not more than two antigens can be directly compared, and those not side by side, it takes some 24 hours longer, and needs rather more antiserum.

The technique employed here is not quite identical with that of the above authors, and will be described in a later publication. Essentially, however, an electrophoretic run is carried out in a 3 mm. layer of barbiturate buffer agar spread on a glass plate. Electrodes of platinum wire are contained in separate compartments: a filter paper bridge connects each of these compartments with a reservoir of buffer, and another connects this reservoir with one side of the agar on the plate; using this method we have experienced no difficulties with *p*H changes, etc. One drop of the serum to be examined, and of a control normal serum (both containing a little bromphenol blue) are added to cups cut in the agar; a current (4–8 mA) is passed, sufficient to move the albumin (as marked by the blue dye) about 4 cm. in 18 hr. Under these conditions the γ-globulin moves backwards with the endosmotic flow, β-globulins remain near the origin but are clearly separated, and the α_2- and α_1- are intermediate. After stopping the flow of current a trough is cut parallel with the direction of movement, 8–12 mm. from the original cup, and this is filled with antiserum. Arcs of precipitate develop centred on the point of maximal concentration of the antigen concerned (Fig. 11).

For testing a pathological serum it is convenient to cut two cups 25 mm. away from one another, one for the test serum and one for normal serum: at the conclusion of the run the trough can then be cut between them, and one can reasonably though not certainly conclude that arcs which are opposite one another represent identical components.

This is of particular value in the examination of the various sorts of γ-globulin. In agreement with Grabar we have found that these are not serologically

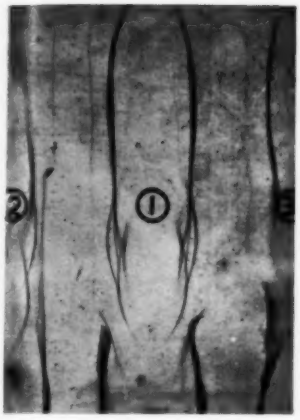


FIG. 11.—Immuno-electrophoretic method. 1, Normal serum. 2, Serum Ha., an undiagnosed case with hypoproteinaemia. 3, Serum Ta., a case of disseminated lupus erythematosus. The fractions are labelled on the right, at approximately the level of maximum concentration, i.e., the centre of the area formed by the precipitate. There is a striking deficiency of γ_2-globulin in 2, and a less marked but still evident deficiency of γ_1-globulin in 3. The α- and β-reactions in 3 do not appear, as the sample of serum was old and these fractions are apt to deteriorate on keeping at 4° C. The albumin and α_1-reactions are unsatisfactory in this plate, for technical reasons.

differentiable; the gamma does not, however, form a true arc of precipitate with antiserum but an elongated and sometimes humped line. It is reasonable to equate these "humps" with the γ_1 and γ_2 globulins (or γ and "β_2" globulins), whose relationship is discussed by Oncley (1953): and the shape of the line can give some evidence as to their relative proportions. Routine analysis of a large number of sera from conditions showing abnormal values for "total γ-globulin," so as to differentiate between these fractions, should supply valuable data as to their pathogenesis. Fig. 11 shows an example of the application of the method to such an analysis.

Discussion

Once an antiserum has been raised and titrated, the immunological method of analysis has some advantage over more usual methods such as paper electrophoresis, though it is not likely to replace the latter for routine use. Its chief disadvantage is its lack of quantitative precision; and although as described above it is possible to adapt it to give a quantitative answer within 10 to 20% this necessitates the expenditure of some thought and possibly repeated tests, a more time-consuming process than a quantitative paper electrophoretic run, by a method such as that described by Hardwicke (1954). On the other hand, there are three great advantages which recommend it as a method for investigating more thoroughly cases shown to be abnormal; its very high sensitivity, valuable in cases of deficiency of a serum protein, its great discriminative power, demonstrating as it can more than 15 different fractions in normal serum as against the five to seven fractions of conventional electrophoretic techniques, and the fact that it depends upon biological or at least biochemical properties of a protein rather than upon the purely physical properties responsible for both the electrophoretic and the ultracentrifugal pattern. In general, immunological analysis by the gel-diffusion method provides a valuable supplementary form of analysis, but cannot displace the usual electrophoretic techniques.

Summary

Methods are described for the laboratory analysis of human serum proteins by immunological and immuno-electrophoretic techniques, carried out in agar. Results in cases of a- and hypo-gammaglobulinaemia, the nephrotic syndrome, myelomatosis, etc., are described. Adaptations leading to semi-quantitative results are suggested.

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ESCHERICHIA COLI O.128 CAUSING GASTRO-ENTERITIS OF INFANTS

BY

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A number of cases of infantile diarrhoea had occurred in the Coventry area over a period of a few months in 1953, and in July a severely ill patient, baby Cigleris, from whom no salmonella or shigella could be isolated, was admitted to hospital. The faeces were cultured on MacConkey agar, and the primary plate sent for the serological identification of *Escherichia coli*. On examination, the plate showed a pure growth of *E. coli* of a single colonial type which was not agglutinated by any of the diagnostic sera in use at that time. Antisera were prepared with suspensions of this strain. Our investigations, which will be described in detail, showed that the somatic antigen did not belong to those already described, *Escherichia coli* O.1–O.126 (Vahlne, 1945; Kauffmann, 1954). There was no relationship to the new type O.127 investigated by Dr. W. H. Ewing (personal communication). A strain was sent to Dr. F. Ørskov at Copenhagen, who confirmed our finding, and assigned the symbols O.128 : B.12 to the somatic and surface antigens.

Source of Material

A few weeks after the Coventry strain of *E. coli* had been received, an outbreak of infantile diarrhoea occurred in the Birmingham Children's Hospital. The strains received were found to be serologically identical with the Coventry type. Altogether 86 strains were identified. Of these, 49 were isolated from infants with gastro-enteritis; 21 strains were isolated from babies whose clinical history is unknown, but who were investigated in relation to institutional outbreaks of infantile diarrhoea; one strain was isolated from the ear swab of a baby who was in a ward where an outbreak was in progress; 12 were isolated from healthy babies, of whom the majority were contacts of clinical cases, but some were from routine admission swabs; one strain came from a case of pertussis with no intestinal symptoms, and two came from healthy adults, contacts of sick babies. Thirteen of the babies with gastro-enteritis were sporadic infections; three of these died. The areas from which strains were received were Coventry, Birmingham, Guildford, Epping, Hertford, Aberdeen, Reading, Southampton, Sheffield, Worthing, Taunton, and Greater London. It is known that outbreaks occurred in institutions in most of these towns.

The age distribution of patients with gastro-enteritis is shown in Fig. 1. Some of these cases occurred after the 49 referred to earlier, as details of age were not always available and we wished to include as many cases as possible. The maximum incidence was in babies under 1 year of age, with the bulk of cases in the 3–6 months' group. Two cases were in older children aged 2 years and 2½ years. The distribution is similar to those found in gastro-enteritis due to other serotypes of *E. coli*.

While this work was in progress, seven of the strains originally believed to be *E. coli* O.128 later were found to be closely related antigenically, but were not identical with O.128. One was isolated from a baby with measles and *Salmonella*

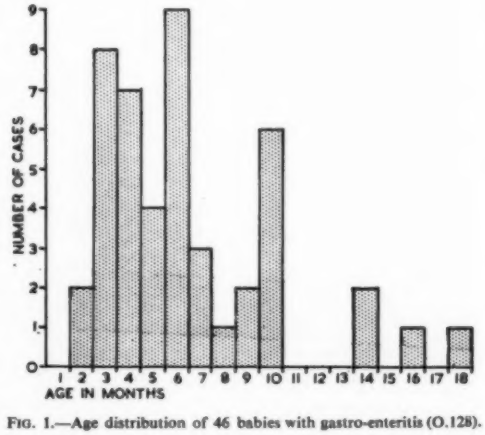

FIG. 1.—Age distribution of 46 babies with gastro-enteritis (O.128).

typhimurium infection; one from a baby with bronchitis but with no intestinal symptoms; four from babies with gastro-enteritis; and one from a healthy contact. Four of the last five strains were from a single nursery outbreak.

Bacteriology

On MacConkey agar incubated at 37° C. all 86 strains produced typical round, smooth, shiny red colonies with an entire edge. Similarly, on blood agar, the organisms showed normal smooth colonial morphology without haemolysis. On keeping the MacConkey plates on the bench it was noticed that the colonies of some strains of *E. coli* O.128 produced an intensely mucoid growth, so much mucoid material being produced that it fell on to the lid when the plate was stored in the inverted position. The Coventry strain Cigleris has been accepted as the type strain O.128, and is designated as strain No. 5594 throughout this communication. The Coventry strain, which produced a mucoid growth, was cultured on a nutrient agar to which different carbohydrates had been added to test their effect on the ability to produce a mucoid growth. The carbohydrates at a concentration of 5% were incorporated in beef extract agar. The plates were thick and dried carefully, so that surface moisture only was removed. All plates were inoculated from a four-hour beef extract broth culture of the Coventry strain. Plates were incubated at 37° C. and 22° C., and examined for mucus production after one, two, and four days' incubation. The results are shown in Table I. Mucoid material was produced more rapidly and in greater amount when cultures were incubated at 22° C. In many instances the mucoid material was first seen at the edge of a well-isolated colony, or at the edge of the confluent growth; later other parts of the culture showed the same change, which was always maximal at the edge of the culture. Although some carbohydrates hastened the speed with which mucus was produced, and, to a certain extent increased its amount, their presence was not essential. An interesting observation was that the ability of the organisms to ferment a particular carbohydrate had no effect on the value of the carbohydrate to assist mucus production. The Coventry strain (Table I) fermented both sucrose and salicin, yet mucus was not produced on the latter medium. In order to check that no change had occurred in the organism, a sucrose agar plate was seeded from growth on a salicin plate, and vice versa. Again mucoid growth occurred on sucrose media but not on salicin. Sixty-three strains were cultured on 5% sucrose agar. After 24 hours' incubation at 37° C. five showed a mucoid change; incubation for a further two days did not increase this number. The cultures were then kept on the bench, and examined after a further 20 days. At the end of this time, four strains of *E. coli* O.128 and two of the serologically related strains still showed no production of mucoid material, six gave a doubtful appearance, and the remaining 51 were positive. Many cultures showed but few mucoid colonies, even though many of the non-mucoid colonies were well isolated. There is reason to believe that this ability to produce the mucoid material is not a property of every culture nor of each colony of a single strain.

TABLE I

MUCUS PRODUCTION BY *E. COLI* O.128 (COVENTRY STRAIN)

	37° C.			22° C.		
	1st day	2nd day	4th day	1st day	2nd day	4th day
Lactose	–	–	–	+++	+++	++++
Glucose	–	–	–	++	++++	++++
Maltose	+	+	++	++	++++	++++
Mannitol	–	–	–	++	++++	++++
Sucrose	sl. +	sl. +	++++	++	++++	++++
Salicin	–	–	–	–	–	–
Dulcitol	+++	+++	+++	+++	++++	++++
Inositol	++	++	+++	+++	++++	++++
Sorbitol	+++	+++	+++	+++	++++	++++
Adonitol	++	++	++	+++	++++	++++
Rhamnose	–	–	–	++	++	++
Trehalose	–	–	–	+++	+++	+++
Starch	? +	? +	–	+	+++	+++
Nutrient agar	+	+	+	++	++++	++++

Eighty-one of the 86 strains of *E. coli* O.128 were motile when grown in soft agar and incubated at 37° C.

Serology

The serology of this group was found to be very complicated, as four antigens required detailed study; the somatic antigen, the heat-labile surface

antigen, the capsular and slime antigen, and the flagellar antigen. Eight representative strains were chosen for detailed study:

5301 .. .. isolated from a sick baby in London
5594 (Coventry strain) " " " " " Coventry
5902 .. .. " " " " " Birmingham
5908 .. .. " " " " " Guildford
5920 .. .. " " " " " Aberdeen
6146 .. .. " " " baby with pertussis in London
5968 isolated from a baby with measles and *S. typhimurium* infection
6080 " " " " " bronchitis

The first six were shown to have identical somatic and surface antigens, whereas the last two were antigenically related to but not identical with the first six.

A number of rabbit antisera were prepared using as inoculum a living suspension of a non-mucoid strain, formolized broth cultures of motile strains, and living suspensions of a mucoid strain for the production of capsular agglutinins.

Somatic and Surface Antigens

The serum prepared from a living suspension was tested, using *Escherichia coli* O.1 to O.126 suspensions, and Dr. W. H. Ewing tested with O.127. No *E. coli* "O" groups were agglutinated except *E. coli* O.48, which was agglutinated to 1% of the homologous titre, and *E. coli* O.87, which showed a trace reaction. Absorption of the serum with

TABLE II
SEROLOGICAL RESULTS ON THE SURFACE AND SOMATIC ANTIGENS

Sera	Suspension					
	Heated	Living	Heated	Living	Heated	Heated
5594	5301 5594 5902 5908 5920 6146	5301 5594 5902 5908 5920 6146	5968	5968	*E. coli* O.48	*E. coli* O.87
Unabsorbed ..	6400	800	6400	400	50	trace 50
Absorbed 5902	< 50	< 25	100	< 25		
" 5301	< 50	< 25	100	< 25		
" 5968	1600	100	< 50	< 25		
" O.48	3200	800	3200	400	< 50	
" O.87	6400	800	6400	400		< 50

E. coli O.48 or with *E. coli* O.87 failed to have a significant effect on the homologous titre. Obviously, the serological relationship to these "O" groups is not close (Table II).

From the agglutination titres and absorption results shown in Table II, one can conclude that strains 5301, 5594, 5902, 5908, 5920, and 6146 are of the same serotype. This was confirmed, as strain 5594 removed all agglutinins from a serum made with strain 5902 and also from a serum made with 5301. It will be seen that strain 5968 was related to serotype 5594 in that the heated suspension was agglutinated to titre, but the living suspension was only agglutinated to 400 (titre 800). Absorption of serum 5594 with 5968 suspension reduced the homologous titre from 800 to 100 and from 6,400 to 1,600, using living and heated suspensions respectively. These results confirmed the conclusion that the somatic and surface antigens of 5968 were not identical with 5594, though closely related. A second strain, 6080, gave reactions identical with 5968.

The remaining 78 cultures were investigated by direct agglutination. Both heated and living suspensions were agglutinated to titre by serum 5594, but six strains were identical with 5968.

The agglutinability of the surface antigen of a living suspension was destroyed by heating to 100° C. for 30 minutes. A suspension of living organisms in normal saline so treated absorbed all somatic and surface agglutinins from the homologous serum. These tests are regarded as sufficient to class the surface antigen as belonging to the B type (Kauffmann, 1954).

"H" Antigens

Two "H" sera were used, the one made from strain 5902 and the second made from a strain 6 S, which is *E. coli* H.2. From Table III it is clear that strains 5594, 5902, 5301, and classical strains Bi.7455 (Vahlne, 1945) and 6 S had identical "H" structures. In addition, reciprocal absorption tests using strains and sera of 5301 and 5594 showed that these two had identical flagellar antigens.

TABLE III
SEROLOGICAL RESULTS ON H.2 ANTIGENS

Sera	Antigens				
	5902H	5594H	5301H	6SH	Bi. 7455H
5902					
Unabsorbed ..	12800	12800	12800	12800	12800
Absorbed by 5594H ..	< 100	< 100	< 100	< 100	< 100
" " 6 S ..	< 100	< 100	< 100	< 100	< 100
" " 5301H ..	< 100	< 100	< 100	< 100	< 100
6 S					
Unabsorbed ..	25600	25600	25600	25600	25600
Absorbed by Bi.7455H	< 100	< 100	< 100	< 100	< 100
" " 5902 ..	< 100	< 100	< 100	< 100	< 100
" " 5301H ..	< 100	< 100	< 100	< 100	< 100

Seventy of the 86 strains were identified as having antigen H.2. Three strains, 6146 and two others, though actively motile, were not agglutinated by H.2 serum, but were found to have H.8. The two related strains 5968 and 6080 and four similar strains were motile, having *E. coli* H.10, and one strain had H.9 and one H.12. Five strains were non-motile.

Capsule Antigen

The relationship of capsule formation to antigenic structure was investigated. Two sera were used, one made by injecting a rabbit with the living non-mucoid strain 5301, and the second by injecting the living mucoid strain 5594 (serum referred to as 5594c).

When cultures were grown at 37° C. overnight on solid media, typical non-mucoid colonies were produced by all strains including 5594. When tested by slide and tube agglutination, using serum made with the non-mucoid strain 5301, the results were those expected—coarse rapid agglutination on slide, and in tube agglutination typical of the somatic, surface, or flagellar type, according to the suspension used.

Six strains, of which 5594 was one, when grown at 22° C. rapidly produced a mucoid growth which did not show a typical slide agglutination using serum made with non-mucoid strain 5301 ; the agglutination was finely granular and occurred rather slowly. This type of agglutination was shown by both the unwashed and washed suspensions. A saline suspension of the mucoid strains, when tested in tube with serum 5301, showed a general turbidity of unagglutinated cells with a deposit of a very few small agglutinated particles. The appearance was entirely different from that seen using serum 5301 and a living suspension of the non-mucoid strain or of 5594 and similar strains grown at 37° C. ; here the agglutinated particles were extremely coarse and the supernatant fluid clear. It is believed that both in the slide and the tube test these agglutinated particles seen with the mucoid strains grown at 22° C. were due to the few non-capsulated organisms (as referred to previously). The capsule formation and mucus production shown by some strains grown at 22° C. have already been described.

Repeating the reactions using the serum made with the living mucoid strain 5594, it was found that suspensions of both the mucoid 5594 and non-mucoid 5301 growth were agglutinated coarsely both on slide and in tube. This serum was absorbed with a living suspension of a non-mucoid strain 5301. The results showed that agglutinins remained which agglutinated only the capsulated mucoid strain 5594 and similar mucoid strains.

Washings from non-mucoid 5301 and mucoid 5594 strains were layered on undiluted serum 5594c, and a positive ring test obtained with both. The washings were then layered on undiluted serum 5301 ; a positive ring test was obtained with the homologous extract but was negative with 5594 extract. Then 5594c serum was absorbed with a living suspension of 5301 and saline washing from the two strains layered on this absorbed serum ; a positive precipitin reaction was given by 5594 extract, but a negative reaction by 5301 extract. Results similar to those of 5594 were given by extracts of the five mucoid strains, the extracts of the non-mucoid strains behaving like those of 5301 (Table IV). The ring test was used with undiluted sera, but with sera diluted 1 : 5 it was found that the capillary tube method gave more satisfactory results.

TABLE IV
REACTIONS OF SURFACE AND SOMATIC ANTIGENS

Antigen		Sera		
		5301	5594c	5594c absorbed with 5301
5301	slide agglutination ..	+	+	−
	capsular swelling ..	−	−	−
	precipitin test	+	+	−
5594c	slide agglutination ..	−	+	+
	capsular swelling ..	−	+	+
	precipitin test	−	+	+

All sera were used at a dilution of 1 : 5.

When suspensions of the non-mucoid strain 5301 in Indian ink were mixed with the homologous antiserum and inspected with the phase-contrast microscope, no change in the appearance of the organism was observed, though agglutination occurred ; similar results were obtained using the same suspension and serum 5594c. With the same technique but with a suspension of the washed capsulated strain 5594 and homologous serum, there was obvious capsular swelling and agglutination of the capsulated organisms. Serum 5594c was absorbed with a suspension of the non-mucoid strain 5301. The absorbed serum caused swelling of the capsule of strain 5594, and all other capsulated strains of the O.128 type, the only type investigated. The absorbed serum caused neither agglutination nor swelling of non-capsulated strains 5301 and others of O.128 type when viewed by phase-contrast microscopy.

From these results it is clear that the capsular antigen differs from the somatic and surface antigens already described.

The thermolability of the capsular antigen in the mucoid variant was studied. Saline suspensions of the growth from solid media of the mucoid-variant and the non-mucoid cultures were each washed twice in saline and the deposit from each centrifugalization resuspended in fresh

saline to the original volume. Each suspension was then divided into two parts and one part of each heated in a water-bath at 100° C. for 30 minutes, cooled, centrifuged, and the deposit resuspended in fresh saline.

The above suspensions were then tested for agglutinability against sera prepared from living suspensions of a normal culture of O.128 and a mucoid variant. A third serum was prepared by absorbing the anti-mucoid serum with a non-mucoid suspension. Table V shows that the heating destroyed the agglutinability of the capsular antigen and also rendered the suspension "O" agglutinable. Similar results were obtained with other mucoid strains of *E. coli* O.128.

TABLE V

EFFECT OF HEAT ON THE AGGLUTINATION OF CAPSULATED STRAIN

Serum	Antigen			
	Washed Suspensions		Heated Washed Suspensions	
	5301	5594	5301	5594
5301	400	<4	1600	1600
5594c	400	200	3200	1600
5594c absorbed with 5301 ..	<25	100	<25	<25

5301 = non-mucoid, non-capsulated strain.
5594 = mucoid, capsulated strain.

Slide Agglutination: Cross Reaction

When doing slide agglutination for the preliminary identification of cultures of *Escherichia coli* O.128, it has been found that organisms later shown to belong to this "O" group were also agglutinated by *E. coli* serum O.55 : B.5 A number of O.55 sera gave this reaction. Sera made with *E. coli* O.128 also gave a poor positive slide test with strains of *E. coli* O.55 : B.5. These cross reactions were tested by the tube technique, but heterologous titres only gave trace readings in low dilution. We believe that these findings indicate a very minor antigenic relationship between these serotypes.

Biochemical Reactions

Twenty strains representing the six varieties of *Escherichia coli* O.128 were investigated in detail, five non-motile, four of the H.2 variety, three of the H.8 variety, one of the H.9 variety, six of the H.10 variety, and one of the H.12 variety (Table VI). A further 66 strains of the H.2 variety were tested on lactose, glucose, maltose, mannitol, sucrose, salicin, dulcitol, and inositol only, and gave results similar to other H.2 strains. The seven strains of the closely related types having H.10 or H.12 differed from the classical strains in their failure to ferment sucrose and raffinose. All strains produced indole, were V.P. negative, M.R. positive, and failed to utilize citrate, liquefy gelatin, or produce urease. All strains produced H_2S slowly when tested by the method described by Clarke (1953). This method is very sensitive; in fact, other members of the Enterobacteriaceae described as H_2S negative will give a positive reaction using Clarke's method.

TABLE VI

BIOCHEMICAL REACTIONS OF *E. COLI* O.128

	O.128 : B.12 " " H.2 " " H.8 " " H.9	O.128 : B.12 : H.10 " " H.12
Lactose, Glucose, Maltose, Mannitol	AG (1)	AG (1)
Sucrose	AG (1–3)	— (21)
Salicin	AG (1–7)	AG (3)
Dulcitol	*AG (1–4)	AG (4–10)
Sorbitol	AG (1–3)	AG (1)
Arabinose	AG (1–2)	AG (1)
Raffinose	AG (1–4)	— (21)
Rhamnose	AG (1)	†AG (2–3)
Xylose	AG (1–3)	AG (1)
Trehalose	AG (1)	AG (1)
Inositol, Adonitol, Inulin	— (21)	— (21)

— = no change. A = acid. G = gas. Figures in brackets = first true fermentation seen.
* One strain failed to ferment this sugar.
† H.12 type failed to ferment this sugar.

Discussion

This new serotype, *Escherichia coli* O.128 : B.12, has been isolated from sporadic cases and outbreaks of diarrhoea of infants from whom no other accepted pathogen was isolated.

The age group affected by *E. coli* O.128 is similar to that affected by other serotypes of *E. coli*. Most of the cases occurred in babies under 1 year, with a concentration of cases in the 3–6 month age group, whereas after 1 year the numbers diminished. Nevertheless, the oldest child was 2½ years old.

Some strains of *E. coli* O.128 : B.12 had a capsule and produced a slime matrix. Although no detailed investigation of the slime and capsule was made, the tests which were done suggested that the two were either the same or very closely related in their serological reactions. On the few tests done, it seems that the capsular antigen was destroyed by heating to 100° C. for 30 minutes, as the heated suspension was no longer agglutinated by the specific serum. This point is being investigated further. The results for the capsular antigen do not agree with those described by Kauffmann for the capsular antigen of the A type. He found it necessary to autoclave suspensions in order to

destroy the agglutinability of the capsular antigen. We have not encountered a description of *E. coli* having a surface antigen of Kauffmann A type in addition to his B type. Therefore, in this paper, only the symbol C is used to avoid confusion with antigens which may be of a very different nature. Our results agree with those of Wilkinson, Duguid, and Edmunds (1954), who state that, "although differing morphologically, the slime antigens resemble the capsular antigens chemically and serologically." Henriksen (1949) has made a study of some mucoid strains, including *E. coli*, showing capsules. He found that the capsular antigen was not the same in all strains.

Six varieties of *E. coli* O.128 : B.12 were found—the non-motile, the H.8, the H.9, the H.10, the H.12, and the most common H.2 varieties. Though few cases of infection due to the non-motile, H.8, H.9, H.10, and H.12 types occurred, there was no difference in the severity of the disease due to these types and the more numerous H.2 type. The H.10 and H.12 types differ slightly from the classical strains in the structure of their somatic and surface antigens, but we believe that the difference is not enough to warrant the use of other symbols, so we shall refer to these as *E. coli* O.128 : B.12 : H.10 and H.12 respectively. The clinical picture of infection with these varieties was similar to the rest. Type H.10 also caused an institutional outbreak.

Summary

A new serotype of *Escherichia coli* is described which has been isolated from cases of diarrhoea of infants occurring sporadically and in outbreaks in different parts of England and Scotland.

The age group affected is similar to that found in infantile diarrhoea due to other serotypes of *E. coli*.

This serotype has been accepted as *E. coli* O.128 : B.12. Of this there are six varieties, the non-motile, the H.2, which is most common, the H.8, the H.9, and the H.10 and H.12. The H.10 and H.12 varieties we include in this group, though the antigenic structure differs slightly from *E. coli* O.128 : B.12. The biochemical reactions of these varieties differ in the failure to ferment sucrose and raffinose.

Some strains produce a capsule and a slime matrix, whose agglutinability seems to be destroyed by heating at 100° C. This finding is still under investigation. The capsular antigen differs from the surface B antigen.

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EPIDEMIC INFANTILE GASTRO-ENTERITIS DUE TO *ESCHERICHIA COLI* TYPE O.128

BY

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At the Birmingham Children's Hospital the Leonard Parsons Block, a separate building with accommodation for 66 infants, was built to prevent the cross-infection of the upper respiratory tract that affected babies when in wards with older children. Upper respiratory cross-infection then virtually ceased in infants, but their segregation now created a new problem, that of epidemic infantile gastro-enteritis.

When it was found that the specific serological types of *Escherichia coli* were constantly recovered from the infants involved in these epidemics of enteritis (Rogers, 1951) a scheme of bacteriological screening was evolved to prevent the admission of any infant harbouring these organisms to the Parsons Block wards. This method of control proved of considerable value and reduced the incidence of infantile gastro-enteritis amongst the babies in the hospital. Details will be published later. A similar scheme has been successfully operated in Brighton (Jameson, Mann, and Rothfield, 1954). It was realized, however, that such a method of control would offer no protection if an infant were admitted excreting a pathogenic type of *E. coli* for which no means of identification was at hand. In October, 1953, such an unrecognized type caused an epidemic which is described below.

On the second floor of the Parsons Block two infants, who had had Ramstedt's operation for congenital pyloric stenosis, both exhibited more post-operative vomiting than usual. On October 5 they both developed diarrhoea. Routine cultures of their faeces failed to show *Salmonellae, Shigellae*, or *E. coli* types O.126 and O.125 (Taylor and Charter, 1952), O.119 (Smith, 1953), O.114 (Charter, 1955; Rogers and Cracknell, 1955), O.111 (Bray, 1945), O.86 (Taylor and Charter, 1952), O.55 (Giles and Sangster, 1948), O.26 (Ørskov, 1951), or other additional types, O.44, E.2581, and E.10732, which are believed to be associated with infantile gastro-enteritis but whose importance is difficult to assess.

By October 11 a further seven infants had developed gastro-enteritis and no presumptive pathogen could be isolated from the faeces of any of them. On October 12 the ward was closed to new admissions. Also on that day faeces from all nine affected infants were cultured and colonies of *E. coli* were subcultured and saved from each, although by this time several of the affected infants were no longer ill. A strain of *E. coli* from the most recently affected baby was employed to immunize a rabbit, and this same strain was also sent to Dr. Joan Taylor at the Salmonella Reference Laboratory, London.

Dr. Taylor found that the organism was specifically agglutinated by an antiserum prepared against a strain of *E. coli* isolated from an infant who had been ill in an epidemic in Coventry in June, 1953 (Taylor and Charter, 1955). With her report she sent some of the specific antiserum and thus saved us from waiting the further three weeks which would have been necessary to complete the preparation of our antiserum. Further work by Dr. Taylor showed that this strain could be put into Kauffman's scheme, being classified by Dr. F. Ørskov and assigned the symbols O.128: B.12 (All future references to it will be as *E. coli* type O.128.) Seven of the nine strains of *E. coli* which had been isolated on October 12 were specifically agglutinated by the antiserum. The two unidentified colonies were from infants who were convalescent from their attack of gastro-enteritis.

As soon as it was available the O.128 antiserum was used routinely in the examination of *E. coli* isolated from any material. This not only disclosed that other babies on the second floor of the Parsons Block were excreting this type but also that it was present in the faeces of infants on the first floor. On October 27 all the infants on the second floor who were excreting the *E. coli* type O.128 were transferred to the first floor of the Parsons Block, which was closed to new admissions. When the second floor was empty it was fumigated

(Rogers, 1954) and afterwards reopened for "clean" cases.

The mode of onset, course of the gastro-enteritis, and the pattern of the spread amongst the infants were very similar to those of the other specific serological types of *E. coli*. It was possible to demonstrate that infants who were excreting no *E. coli* type O.128 when they were admitted later excreted this type and, when still doing so, began to suffer an attack of gastro-enteritis.

The clinical picture of the affected infants varied considerably, but often they showed symptoms of vomiting for two or three days before the onset of the diarrhoea; when the diarrhoea started it was not uncommon for the vomiting to cease. Some cases had a pyrexia of up to 102° F. Several cases were given prophylactic sulphadiazine as soon as their faeces contained *E. coli* type O.128, but too few were treated in this way for any conclusions to be reached. Only one fully breast-fed baby excreted the organism and no untoward symptoms appeared, but, as he was given prophylactic sulphadiazine and no other breast-fed babies became excretors, no conclusion can be drawn from this one case.

Between October, 1953, and January, 1954, strains of *E. coli* type O.128 were isolated from 39 infants in the Parsons Block. Also, two babies with enteritis attended the Out-Patient Department and were found to be excreting these organisms; one of them had never been in an institution, and the other was 3 weeks old and had been born in a local maternity hospital, where no source of infection could be discovered. During this period *E. coli* type O.128 must have been present amongst the babies of Birmingham, as when the antiserum was sent to a local fever hospital it was found that several babies there with infantile gastro-enteritis were excreting this type, which caused considerable cross-infection in the wards at the hospital.

In addition, on October 28, 1953, one strain was isolated in pure culture from a middle ear infection in a 6-year-old out-patient. No *E. coli* type O.128 could be recovered from this child's faeces on November 2. This is the first time we have ever isolated any of these specific serological types of *E. coli* from a middle ear infection, although over the last five years we have tested more than 200 strains found in aural discharges.

Nearly a quarter (24.1%) of the babies who excreted *E. coli* type O.128 had no symptoms of gastro-enteritis; their average age was 30 weeks. Just under half of the infants (47.1%) suffered from mild gastro-enteritis and their average age was 18 weeks. More than a quarter (29.1%) of the babies had severe gastro-enteritis; 8%, with an average age of 12 weeks, were treated with intragastric drips, and 21%, whose average age was 8 weeks, required intravenous nourishment. Seven babies were well when discharged from hospital and had to be readmitted with gastro-enteritis. There was one death from the gastro-enteritis.

The direct wet smear examinations of the faeces of the babies with acute gastro-enteritis due to type O.128 were unusual. They contained much mucus and a moderate number of pus cells, as well as an occasional red blood cell.

Five specimens of vomit from ill babies were cultured, but none was found to contain any *E. coli* type O.128.

We would like to record our gratitude to the staff of the hospital, who have encouraged this work.

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RECURRENT INFECTIONS BY A STABLE DWARF-COLONY VARIANT OF *STAPHYLOCOCCUS AUREUS*

BY

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Small-colony variants of *Staphylococcus aureus* are sometimes seen among the normal colonies obtained in primary cultures from pus. On subculture these small colonies usually yield growths of typical large colonies. Many workers, however, have obtained stable small-colony variants *in vitro* by selecting them from cultures grown in media containing inhibitory salts, antiseptics, and antibiotics, as summarized by Chain, Florey, and Jennings (1949 ; see also Browning and Adamson, 1950). Wise and Spink (1954) also review the literature on G (dwarf-colony) variants and have themselves produced them *in vitro* and isolated them from eight patients, seven of whom were being treated with antibiotics or sulphonamide. Hale (1951) was the first to report a pure growth of a dwarf-colony variant of staphylococcus in primary culture from an abscess ; it gave the normal large-colony type of growth in the presence of 10% CO_2. Two similar cases were reported by Sherris (1952). A similar case, but with recurrent infections, is reported in this paper.

Clinical Description

A male second-year medical student aged 21 years had a history of nine pulp infections of the fingers during January to April, 1954. Intramuscular crystalline penicillin was used without effect to treat the first, the lesion progressing to the formation of an abscess, which healed rapidly, however, after surgical incision, drainage, and treatment with oral chloramphenicol. Later lesions also resolved satisfactorily with chloramphenicol (January–April, total probably 30–40 g.), including the ninth, which required surgical drainage early in April. At this time a tenth lesion continued to progress although the patient was still taking chloramphenicol. Eight days after it first appeared this lesion was incised by a practitioner, and a day later the patient came for the first time to the hospital out-patient department with an open sore on the medial side of the middle phalanx of the left index finger. Thick pus was present in the base of the lesion and the whole finger was painful, stiff, and swollen, with an inflammatory swelling spreading on to the dorsum of the hand. When the patient arrived at hospital the pus was taken for the first time for bacteriological investigation. Treatment was continued with splinting, chloramphenicol, and later by a course of aureomycin (total 8 g. in four days), but it was not until June, eight weeks later, that the lesion had resolved and a crust had formed. On the three occasions on which swabs were taken from this lesion for bacteriological examination, twice in April and once in May, pure cultures of the dwarf-colony variant *Staphylococcus aureus* described below were obtained.

The patient was then free from septic lesions until the last day in August, when another whitlow developed while he was on holiday, working at forestry. He had a few septic spots on his legs, buttocks, and neck in September, but these healed rapidly when treated with chloramphenicol and penicillin for a few days. In mid-October, when he returned to Glasgow for classes, he reported once more to the Western Infirmary ; the whitlow, now six weeks old, still had pus in the base of the wound, and a swab from it gave a pure heavy culture of dwarf-colony staphylococci. At this time he had not had any antibacterial drugs for more than a month ; a boil which was rapidly developing in his neck ruptured a few days later, and the swab from it also yielded on culture a pure growth of the dwarf-colony staphylococcus. The whitlow, like the previous one, required eight weeks to heal.

Bacteriological Findings

The organism to be described was obtained in pure culture on seven separate examinations: three times from swabs of pus from the first lesion examined, twice from the last whitlow, and twice from the boil in the neck.

Morphology and Staining.—Smears from pus and from cultures showed typical clusters of Gram-positive spherical staphylococci 0.7–0.9 μ in diameter.

Cultural Characters and Physiology.—After 18 hours' growth at 37° C. on 8% horse-blood-agar or nutrient-agar plates, the colonies were almost invisible to the naked eye, but by 24 hours were

just visible as smooth, shiny, colourless, translucent discs, measuring 0.01 to 0.1 mm. in diameter (Figs. 1 and 2). When these dwarf colonies were heaped up on the medium the aggregate was opaque and creamy, and after exposure to daylight at room temperature for a few hours it developed the golden yellow of a typical *Staph. aureus*. After 96 hours' incubation in air at 37° C., some discrete colonies had grown up to 1 mm. in diameter, as did a few in the more heavily grown parts of the plates. During 40 serial subcultures made at one- or two-day intervals with loopfuls of organisms from the butt the colonial appearances did not alter. The larger colonies which were subcultured also produced only the minute dwarf colonies after 24 hours' incubation.

At room temperature, on horse-blood-agar or nutrient-agar plates, growth became visible only after 72 hours. In anaerobic conditions (McIntosh and Fildes jar) at 37° C., colonies on plates were just visible after 24 hours. When subcultures on blood agar were incubated for 18 hours at 37° C. in an atmosphere of air with 10% CO_2, only large

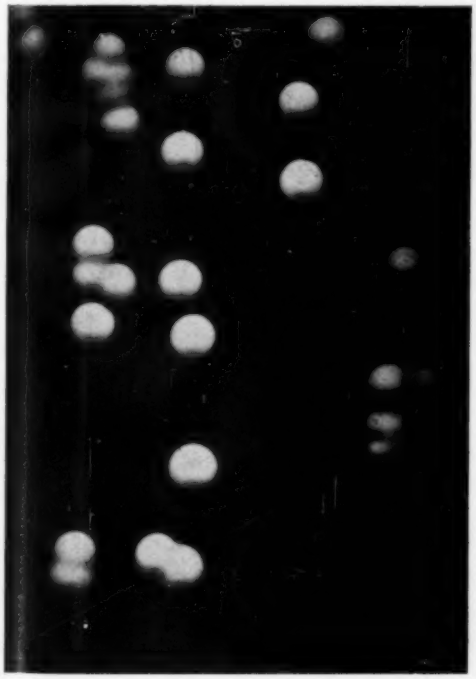

FIG. 1.—Normal staphylococcal colonies on a blood-agar plate; 18-hour culture at 37° C. in air for comparison with Figs. 2 and 3. × 8 (approx.).

FIG. 2.—Dwarf staphylococcal colonies on a blood-agar plate; 18-hour culture at 37° C. in air. × 8 (approx.).

colonies, 1.5 to 2 mm. in diameter, typical of *Staph. aureus* were obtained. Under these conditions the colonies were surrounded by zones of complete haemolysis on 8% rabbit blood agar and on horse blood agar. When these large colonies grown in CO_2 were subcultured in air, pure cultures of dwarf colonies were once again grown. On plates streaked both with normal and dwarf staphylococci, satellitism of the dwarf-colony variant around the normal staphylococci was observed (Fig. 3). When grown on slopes in bottles with the caps tightly screwed down, growth became heavy like that of a normal *Staph. aureus*—no doubt because the CO_2 concentration rose from the organism's own metabolic processes (Hale, 1951; Sherris, 1952). In meat-extract broth a uniform turbidity was produced after 18 hours at 37° C. Only slight growth was obtained in peptone water. Subcultures on nutrient agar of five-day-old fluid cultures gave pure growths of minute colonies.

In 24 hours at 37° C., the organism fermented glucose, lactose, sucrose, and maltose, with the production of acid but not of gas; mannitol was

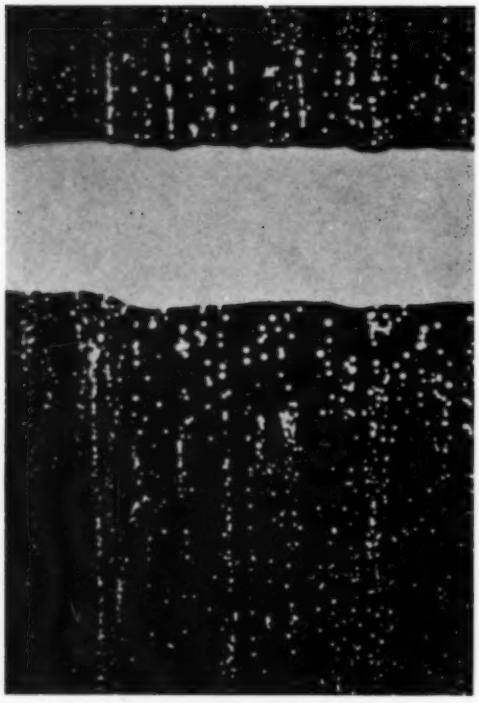

FIG. 3.—Dwarf-colony variant of *Staph. aureus* on a blood-agar plate yields larger colonies near an inoculum (white band near top of figure) of normal staphylococci; 18-hour culture at 37° C. in air. × 8 (approx.).

attacked in the same way only after 72 hours' incubation. The organism did not ferment xylose, glycerol, dulcitol, or salicin; it acidified litmus milk after 72 hours. It produced saccate liquefaction in a gelatin stab culture after two weeks at room temperature, but did not liquefy coagulated serum. The organism produced catalase and urease. Methyl-red and Voges-Proskauer reactions, citrate utilization, nitrate reduction, and indole production were negative. The organism was repeatedly tested and always found to produce coagulase. The organism (grown in CO_2) did not react with known phages.

Sensitivity to Antibiotics.—With sensitivity test tablets ("sentests," Evans) on heavily inoculated plates grown in air, the organism was completely resistant to penicillin (0.5 I.U.), and almost completely resistant to chloramphenicol (40 μg.), but sensitive to streptomycin (20 μg.) and aureomycin (10 μg.).

Further Mutation in Old Cultures.—When plate and slope cultures were left in a cupboard at room temperature for eight weeks and were then subcultured, only small colonies appeared, but these colonies all remained small even after prolonged incubation in air, or in air with 10% CO_2; they were no longer *aureus* when heaped up; but they were still strongly coagulase positive.

Isolation from Patient's Nose.—The stable dwarf staphylococcus was also grown from swabs taken on various occasions from the patient's nose, no normal staphylococcal colonies being found at any time. During the mucopurulent stage of a common cold the staphylococci were isolated only from cultures incubated in 10% CO_2; at this time on plates incubated in air staphylococcal colonies could not be recognized in the heavy mixed flora of *Strep. viridans* and Gram-negative diplococci. The staphylococcal colonies isolated in an atmosphere of CO_2 gave rise only to minute colonies when subcultured in air.

Discussion

The dwarf-colony variant *Staphylococcus aureus* described was grown pure in primary cultures of pus from two whitlows and a boil. It is of interest that the whitlows from which it was isolated persisted for eight weeks in spite of adequate drainage and, in the first lesion, the use of antibiotics. In contrast, the patient's other lesions all healed rapidly with antibiotic and other appropriate treatment. This was true also of the ninth lesion, which developed along with that which first yielded the dwarf staphylococci and also required incision. Only one lesion remained when the patient first attended hospital, and it is unfortunate that this alone of the 10 lesions which developed between January and April was available to us for bacteriological examination.

Hale does not mention previous antibiotic treatment in his case; Sherris's two cases were both treated by intramuscular penicillin for 24 hours before the swabs were taken; in both only dwarf colonies grew and both were sensitive to penicillin, the lesions healing in five and 12 days. But in view of other workers' findings that stable dwarf-colony variants can be produced in the presence of antibiotics, previous therapy may have been responsible for the production of our dwarf strain; if so it is possible that this type of infection may be more common than is realized. Unless the bacteriologist is aware of the possibility that dwarf variants may grow in pure culture—without a single typical staphylococcal colony—he may easily fail to recognize the organism as a pathogenic *Staphylococcus aureus*. This is particularly

worth remembering in examining such specimens as urine, or throat-swabs and sputa, where the presence of the minute colonies may be missed altogether, particularly in a mixed flora. Stokes (1955) recommends great extension of the use of cultures in CO_2 in routine bacteriology.

Our strain of dwarf-colony variant seems to have a metabolic defect similar to that of the strains described by Hale and by Sherris. As originally isolated, our strain required CO_2 to develop normally and produced minute colonies when grown only in air. Hale showed that his strain of dwarf staphylococcus when grown in air was deficient in the dismutation of pyruvate, and postulated that this was due to failure to develop an enzyme produced in an atmosphere containing CO_2. The satellitism which we observed is perhaps due to diffusion of the enzyme postulated by Hale from the typical actively growing staphylococcal colonies, or merely to diffusion of CO_2 in the medium. Experiments showed that the small size of the colonies was not due to lack of any of the known vitamins. Even the smallest dwarf colonies on the plate in Fig. 3, which was inoculated with a normal as well as with the dwarf staphylococcus, were very much larger than those on a similar plate without normal staphylococci (Fig. 2), presumably due to the accumulation of CO_2 in the atmosphere and agar from the heavy growth. The larger satellite colonies did not grow as large as normal colonies even on prolonged incubation.

In recent articles, Spink (1954) and Wise and Spink (1954) state that in the absence of antibiotic small staphylococcal colonies revert to large colonies, but this was not so in our case. Pure dwarf colonies were isolated from various lesions and also from the nose of our patient between early April and late October, showing that a nasal carrier may retain even an abnormal staphylococcus for a substantial period (almost seven months). Moreover, unlike the occasional small colonies that grow among large colonies on plates of normal staphylococci, these dwarf colonies were stable *in vitro* for at least 40 subcultures on solid media. Wise and Spink state that G variants lose virulence and remain viable in animal tissues without producing signs of infection. From the descriptions in previous papers and the first "dwarf-staphylococcal lesion" in our case, the dwarf staphylococci may have been surviving mutants from normal staphylococcal populations, but there is very strong evidence that the dwarf-colony staphylococcus was the primary pathogen in the last two lesions in our case, certainly in the boil on the neck.

Summary

In a patient who had recently had multiple whitlows treated with antibiotics, pure growths of a dwarf-colony variant of *Staphylococcus aureus* were obtained repeatedly in primary cultures of pus from two whitlows and a boil, and also from the patient's nose, between early April and late October. This is confirmation that a carrier keeps the same staphylococcus for many months, even if it is an "abnormal" staphylococcus. There is strong evidence that these dwarf-staphylococci were the primary pathogens at least in the last two lesions; the chronicity of the two whitlows (each required eight weeks to crust over) is striking.

Unlike the occasional small colonies seen in plates of normal staphylococci, these dwarf-colony staphylococci were stable both *in vitro* for 40 subcultures, and *in vivo* in the patient for almost seven months. The metabolic defect was overcome while the organism was grown in an atmosphere of air with 10% CO_2. Primary cultures in 10% CO_2 made it possible to isolate the dwarf staphylococcus from mixed cultures from the nose during the mucopurulent stage of a common cold. After cultures had been left at room temperature for eight weeks, the organism had further changed in that, although it still grew only as dwarf colonies and produced coagulase, the growth was no longer *aureus* when heaped up, nor would the colonies become typically staphylococcal when incubated in an atmosphere of air with 10% CO_2.

Bacteriologists engaged in routine work will realize that dwarf staphylococci in pure culture may readily be overlooked. This may be of some importance in view of the evidence cited that pure growths of dwarf-colony staphylococci can be produced *in vitro* by cultivation of normal staphylococci in antiseptics or antibiotics.

We thank Dr. R. E. O. Williams, of the Staphylococcal Reference Laboratory, Colindale, for examining our cultures; Mr. Athol Parkes, of the Orthopaedic Department of the Western Infirmary of Glasgow, for his co-operation in allowing us to examine his patient; and Mr. G. Kerr for the photographs.

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STUDIES ON A CO_2-DEPENDENT STAPHYLOCOCCUS

BY

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A dwarf strain of *Staphylococcus aureus* was isolated in pure culture from styes on the eyelid of a young woman. Incubation of pus cultured on horse blood agar in 5% CO_2 yielded a typical golden staphylococcus, coagulase and catalase positive and penicillin sensitive, but on duplicate plates in air the growth was of tiny white colonies only 0.25 mm. in diameter. These were penicillin-sensitive Gram-positive cocci, but the catalase and coagulase tests were negative. Subcultured with added CO_2, typical 2 mm. colonies of *Staphylococcus aureus* developed, but in air all grew as dwarfs. This coccus was of bacteriophage type 52A.

The patient, who had used penicillin ointment, was now given systemic penicillin and her styes healed, but the dwarf staphylococcus was present in nasal swabs a month later. Swabs from her mother, who had a palmar abscess and then a sore throat, also yielded a dwarf staphylococcus of type 52A which required added CO_2 for normal growth.

Experimental Observations

The CO_2 requirements of these dwarf cocci were studied in parallel with a "normal" staphylococcus of the same bacteriophage type (52A). Some effects of altering the atmosphere and temperature of incubation, and the *p*H and composition of the medium, were observed. In order to standardize conditions all cultures were incubated for 20 hours on single nutrient agar plates in 3.3 litre air-tight jars (unless otherwise stated). Atmospheres are described at the start of incubation because of changes in the gaseous content of the jars during incubation. O_2 and CO_2 gas were obtained from cylinders.

Coagulase tests were done with standard solid inocula, since broth cultures masked variations. A 2 mm. wire loaded from a plate culture was emulsified in 0.5 ml. of 1 in 5 human plasma and incubated at 37° C., the tubes being examined half-hourly for four hours and finally after 24 hours.

In the record which follows intermediate stages are omitted and both strains of dwarf coccus are described together because they gave identical results. Colonies termed "recognizable" gave positive catalase and coagulase reactions. Growth described as "normal" corresponded to the control staphylococcus in air. It was found that the diameter of the cocci was about 0.9 μ whatever the size of the colony. The pleomorphic forms described by Wise and Spink (1954) were not encountered.

Table I shows the relation between the size of the colonies of the dwarf coccus and their reactivity. Small colonies of the control were obtained by short incubation.

Atmospheric Carbon Dioxide and Water Vapour, and Times of Incubation.—In jars of air growth of cultures of the dependent coccus was unrecognizable, but when the air was saturated with water vapour (boiled and distilled) small catalase-positive colonies appeared. Fully normal growth did not occur in saturated atmospheres without 0.75% CO_2 or in dry jars without 1% or more of CO_2 throughout incubation, but six hours in CO_2 during incubation enabled some catalase and coagulase to be produced. Even in CO_2 the first eight hours of growth were abnormally slow. Cultures failed when CO_2 was removed by incubating plates above potassium hydroxide solution, whereas the control staphylococcus grew quite well.

Incubated for five days in air, a colony of 2 mm. diameter was attained after 96 hours, whereas the control reached 4 mm. in 48 hours. In a wet jar of air 4 mm. was attained, but only after 96 hours. With added CO_2 the dwarf behaved like the control. Some sealed cultures stored for months grew well in air on primary subculture but then reverted to the dwarf form.

TABLE I

CHARACTERISTICS OF DIFFERENT-SIZED COLONIES OF THE CO_2-DEPENDENT STAPHYLOCOCCUS COMPARED WITH THOSE OF THE NORMAL CONTROL

Description of Colony and Average Diameter	Dwarf Less than 0·5 mm.	Dwarf 0·5 mm.	Recognizable 0·5–1·5 mm.	Normal 1·5–2 mm.	Normal 2–4 mm.
Pigment	None	None	Poor	+	+++
Haemolysis (horse blood)	,,	None	Slight	+	+
Coagulase (by solid inoculum method)	,,	Partial at 4 to 24 hr.	1½–4 hr.	½–1½ hr.	Less than ½ hr.
Catalase	None	None	+ Variable	+	+

At comparable colony sizes the control produced more pigment, haemolysin, catalase, and coagulase than did the CO_2-dependent coccus.

Oxygen.—With 1% or more of CO_2 the dwarf coccus grew like the control under atmospheric conditions. Under anaerobic conditions in a MacIntosh-Fildes jar it grew like the control to a diameter of 0.5–1.5 mm., but when KOH or soda lime was added to absorb CO_2 the dwarf coccus failed to reach 0.1 mm. in diameter, while the control reached 0.5–1.5 mm.

Bicarbonate Solutions.—Enough CO_2 for the dwarf staphylococcus to grow normally was released by 100 ml. of 1% aqueous solution of $NaHCO_3$ placed at the bottom of a jar. It was calculated that, at 37° C., 0.75% of free CO_2 would be present in such a jar. Even better growth occurred over a 1.5% bicarbonate solution, corresponding to 0.9% CO_2, but no further improvement resulted from increasing concentration above this level.

H-ion Effects.—Experiments were repeated using media adjusted to *p*H 8.4 and to *p*H 6. The growth of the dwarf organism was not affected and in no case was the final *p*H of the medium outside these limits.

Temperature.—In air the dwarf coccus grew better at 30° C. (diameter 0.5–1.5 mm.) than at 37° C. (diameter less than 0.5 mm.), although in CO_2 it, like the control, grew best at 37° C. (2 mm.).

Some of these details are presented in Table II, in which the results were obtained after 20 hours' incubation on single nutrient agar plates in 3.3 litre sealed jars.

Carbon Dioxide in the Medium

Baryta absorption tests showed that, while nutrient agar (Davies N.Z. powder, autoclaved once) incubated under standard conditions itself released variable amounts of CO_2, greater amounts of CO_2 were released by media to which "analar" sodium bicarbonate was added at 56° C. (Such plates were dried at 37° C. because at higher temperatures much CO_2 was lost.)

TABLE III

GROWTH ON BICARBONATE-CONTAINING MEDIUM

Concentration of $NaHCO_3$ in the Medium (mg. per 100 ml.)	Diameter of Colonies of Dwarf Staphylococcus (mm.)
None	<0·5
172	0·5–1·5
200	1·5–2 normal
>350	<0·5

TABLE II

SUMMARY OF RESULTS OF EXPERIMENTS VARYING THE CONDITIONS OF GROWTH OF THE CO_2-DEPENDENT STAPHYLOCOCCUS

Conditions at the Start of Incubation						Diameter (mm.) of Colonies at End of Incubation
CO_2 from Cylinder			$NaHCO_3$ Strength of Solution (100 ml. inside Jar)	O_2 in Dry Jars	Time (Hr.) in CO_2 before Transfer to Air (1% to 10% CO_2)	
In Dry Jars	In Anaerobic Jars	In Moist Jars				
*Nil to atmospheric	*Nil			100%	2 and 4	0 to 0·5—unreactive dwarf colonies†
0·5% and 100% ..	Traces, 1% and upwards*	0·03% to 0·5%	Nil to 0·5% (0·4% CO_2)	Nil* (with 5% and up to 100% CO_2)	6 and 8	0·5–1·5—reactive colonies
1% to 10% ..		0·75%	1% and 1·25% (0·75–0·9% CO_2)	10–20% (with 5% CO_2)	20	1·5 to 2—normal
		1% to 10%	1·5% or more (1·1% CO_2)			2 to 4—large

The control staphylococcus grew full-sized reactive colonies except where an asterisk indicates small colonies between 0·5 and 1·5 mm. in diameter.

Added $NaHCO_3$, 200–300 mg. per 100 ml. of medium, enabled the dwarf coccus to grow to normal size on unsealed plates in the aerobic incubator. Higher concentrations had an inhibitory effect, and more than 150 mg. of $NaHCO_3$ in blood agar plates caused haemolysis.

Sealed plates grew better than unsealed plates in proportion to the number of hours for which the seal was left intact.

Seals were made with a " plasticine " which did not of itself release appreciable CO_2 (see Wilson, 1930).

Increasing the volume of medium improved growth, and, when one-third or more of the volume of any sealed container was occupied by ordinary nutrient agar, growth was normal.

Other Medium Supplements

Growth was no better on blood, chocolate, serum, egg, glucose, lactose, mannitol, or Bordet-gengou media than on nutrient agar. Bacto-difco yeast extract was also ineffective.

Discussion

Dwarf variants of *Staphylococcus aureus* were selected from cultures in antiseptics by Browning and Adamson (1950) and were later isolated from an abscess by Hale (1951), who found them to be CO_2-dependent. Sherris (1952), who described dwarf staphylococci of types 3C and 52A in two patients, thought that the variation might have been induced in one by penicillin, and Wise and Spink (1954) have selected dwarf variants *in vivo* from patients treated with antibiotics. The isolation of an identical organism from an untreated contact of our first case suggests that, whatever their origin, CO_2-dependent organisms are transmissible and may be primary pathogens. The importance of their identification is obvious.

It is unexpected that so definite a need for CO_2 should be satisfied by 1%, for organisms in need of this gas may demand much higher concentrations (Huddleson, 1943). However, Holm (1954) has reported *Actino bacillus* strains which needed CO_2 yet were satisfied by 0.5%, and he too found humidity an advantage.

In the case of a dependent meningococcus CO_2 has been replaced by adding 0.01% of " bacto "-yeast extract to the medium (Tuttle and Scherp, 1952), but this did not satisfy our staphylococcus.

The CO_2 requirement of the dwarf staphylococcus was clearly lower in a water-saturated than in a dry atmosphere. Possibly the entire H_2CO_3 molecule is better absorbed than CO_2, for this is true of other biological cells (Höber, Hitchcock, Bateman, Goddard, and Fenn, 1945).

When metabolism was slowed by anaerobiosis or cool incubation, the dependent coccus grew like the normal control at CO_2 concentrations below 1%; but if either aerobic or anaerobic cultures were exposed to KOH such further CO_2 deprivation prevented visible growth of the dwarf coccus without much reducing the colony size of controls; thus the dwarf coccus required abnormal quantities of CO_2 even for anaerobic respiration. After growth in 10% CO_2 the pyruvate dismutation of Hale's strain proceeded normally. His theory that therefore the dependent staphylococci stored enzymes only while growing in CO_2 might explain our findings.

The catalase and coagulase reactions of Hale's strain were not described. When our dwarf staphylococcus was grown without adequate CO_2 both were negative, resembling the G strains of Wise and Spink (1954). Coagulase, catalase, haemolysis and pigment production per unit weight of cells of the dwarf coccus were all increased by increasing the number of hours of growth in 1% or more of CO_2.

Wilson (1931a and b) stated that bicarbonate did not enhance the growth of *Br. abortus*. Andersen (1951), however, found that it did replace CO_2 in sporulating cultures of *Cl. botulinum*, and our medium containing 0.3% sodium bicarbonate provided enough CO_2 for the dependent staphylococcus to grow normally in air. Since there is no evidence that the HCO_3 radicle is utilized directly the success of this medium simply reflects the tolerance of an alkaline *p*H and the low critical threshold for CO_2 of the organism.

Summary

Carbon-dioxide-dependent strains of a haemolytic *Staphylococcus aureus* type 52A were isolated in pure culture from two members of one household.

These organisms were not recognizable as staphylococci on plate cultures incubated for 20 hours in air, for they grew as tiny white G-colonies which were catalase and coagulase negative and might have been streptococci.

With 1% or more of CO_2 growth was like that of a normal control *Staphylococcus aureus*.

Less CO_2 (0.75%) was needed in humid atmospheres.

The coccus grew better at 30° C. than at 37° C. in air, but in CO_2 it grew best at 37° C.

Catalase and coagulase production varied with colony size, which depended upon the amount of CO_2 available in terms of time and concentration.

Sodium bicarbonate was a useful source of CO_2, either in solution within the incubating jar or as 0.3% of the medium.

I wish to thank Colonel H. Bensted and Professor G. S. Wilson for their advice and criticism, Mr. T. Nash for designing the experiments with bicarbonate solutions and much other practical help, and Miss J. Rippon for bacteriophage typing and for supplying the control staphylococcus strain.

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THE COMPLEMENT-FIXATION TEST IN THE DIAGNOSIS OF HERPES SIMPLEX INFECTIONS

BY

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As infection with herpes simplex virus appears to be more common than was formerly supposed, laboratory confirmation of the role of this virus in lesions of the mouth, central nervous system, skin, and liver is being sought more often.

The simplest laboratory method for diagnosis is by the complement-fixation test, and a number of different methods of making antigen have been recommended since 1929, when Bedson and Bland described their method for this procedure. The correlation between the complement-fixing and neutralization tests has been shown to be very close if not always absolute (Hayward, 1949; Dudgeon, 1950; Holzel, Feldman, Tobin, and Harper, 1953).

A comparison of some of these methods was made during the performance of routine tests for herpes simplex infection and it was thought that a report of this experience might be of interest.

Methods of Preparing Antigens

Two strains of virus were used, "E.W.H." isolated from a patient with encephalitis, and "McC," which was egg adapted. Both were used for producing antigens from the chorio-allantoic membrane, the E.W.H. strain for guinea-pig, mouse, and rabbit inoculation, and the McC strain for yolk sac inoculation. Control antigens were made in each case from the corresponding normal tissue.

A. Chorio-allantoic Membrane (C.A.M.) Antigens.—Following inoculation with a dose of virus which produced confluent lesions after 48 hours, the chorio-allantoic membranes were harvested and antigen prepared by the methods of the following:

(1) *Hayward* (1949).—The membranes were ground in a pestle and mortar with 0.5 ml. of 20% Hartley broth in normal saline, kept at room temperature for two hours, and centrifuged at 2,500 r.p.m. for 15 minutes; the supernatant was removed and used as antigen.

(2) *Dudgeon* (1950).—Infected membranes were ground with 2 ml. of diluent per membrane, frozen and thawed six times, and centrifuged in an angle machine at 5,000 r.p.m. for 30 minutes. The supernatant was used as antigen.

(3) *Modification* (1) *of Hayward's Method.*—Before centrifugation the C.A.M. extract was frozen at −30° C. for 10 days.

(4) *Modification* (2) *of Hayward's Method.*—Chloroform, 0.05 ml., as recommended by Hoyle (1948) for preparing influenza complement-fixing antigen was added to each millilitre of antigen, the mixture shaken, and left at 4° C. overnight. After light centrifugation the supernatant fluid was used as antigen.

(5) *Casals* (1949).—Infected membranes were treated by the method which is described later (section C2).

B. Guinea-pig Pad Antigens.—Two methods were used.

(1) *Bedson and Bland* (1929).—Four to five days after intradermal inoculation of the footpads of both young and adult guinea-pigs, the animals were killed and the pads removed. They were cut into small pieces and ground, using glass beads and 4–5 ml. normal saline per pad as diluent. After being kept at room temperature for three to four hours, the supernatant was pipetted off and used as antigen.

(2) *Modification of Bedson and Bland's Method.*—Antigen thus prepared was treated with chloroform as already described (section A4).

C. Mouse Brain Antigens.—Casals' two methods were used.

(1) *Casals and Palacios* (1941).—Brains of herpes-infected mice were emulsified in 10 volumes of normal saline containing 2% inactivated guinea-pig serum. The emulsion was held at 4° C. overnight and centrifuged at 2,500 r.p.m. for 30 minutes. After freezing and thawing the supernatant five times it was spun at 4,500 r.p.m. for 30 minutes in the angle centrifuge. The supernatant of this spinning was used as antigen.

(2) *Casals* (1949).—Brains of infected mice were removed and weighed, 20 volumes of acetone added, and the mixture emulsified in an "atomix" blender for two minutes in the cold. After spinning in a horizontal centrifuge at 1,500 r.p.m. for one minute, the supernatant was replaced with 20 volumes of

acetone, then 20 volumes of acetone-ether mixture followed by two 20-volume changes of ether alone. Between each change the mixture was allowed to stand for 20 minutes at room temperature with occasional shaking and then centrifuged. After the last ether extraction the material was dried *in vacuo* and the residue redissolved in 3 volumes of normal saline and kept at 4° C. overnight. After centrifugation at 10,000 r.p.m. for one hour the supernatant was removed and used as the antigen.

D. Allantoic and Amniotic Fluid Antigens.—Two processes were needed.

(1) *Gajdusek, Robbins, and Robbins* (1952).—After inoculation of the yolk sac of 8–9-day-old developing chick embryos with a dose of virus which allowed most of the eggs to survive for four to six days, the allantoic and amniotic fluids were harvested and used directly as antigen either separately or after pooling. If a large amount of urates were present the fluids were dialysed in M/100 phosphate buffer overnight.

(2) *Hayward* (1949).—Infective C.A.M. extracts were inoculated directly into the allantoic cavity and the fluid harvested after three to five days. Amniotic fluid antigens were not obtained by this form of inoculation.

Technique of the Complement-Fixation Tests

For routine antibody titrations the optimal antigen dilution was obtained by a chessboard titration using twofold dilutions of antigen and immune serum. For determining the effect of chloroform and other agents on the antigen, dilutions of antigen were titrated against a constant volume of a specified dilution of serum, which was four times that volume which gave 100% complement fixation with the optimal dilution of antigen.

The five-volume test was used with 0.2 ml. of each component. Complement was guinea-pig serum diluted to give two M.H.D.s per unit volume. Fixation proceeded overnight at 4° C. The haemolytic system was 2% sheep cells + 5 M.H.D. haemolysin. Results were read after 30 minutes in the water-bath at 37° C. End-points were taken as 75–100% fixation in most instances.

Results

The allantoic and amniotic fluids and the extracts of C.A.M. and guinea-pig pads, in which neither chloroform nor acetone and ether were used in their preparation, yielded antigens of adequate concentration for routine complement-fixation tests with herpes simplex virus. With both C.A.M. and pad antigens, chloroform reduced the yield of antigen at least fourfold and the acetone and ether even more than this when compared with untreated controls. The yield from infected mouse brains was very poor, the extracts having to be used undiluted in order to obtain adequate fixation, and some of these preparations tended to be anti-complementary at this concentration. These findings are similar to those of Garabedian (1953) with his ether-extracted antigens.

However, with similar preparations prepared as controls from normal, non-infected materials, 23 of 102 sera (22.6%) reacted non-specifically with C.A.M. and seven of 49 (14.3%) with guinea-pig pad while none of 109 sera reacted in this way with allantoic or amniotic fluid (Table I). Fifteen of these sera were tested in a dilution of 1 in 4 for non-specific fixation with preparations from both the chorio-allantoic membrane and the guinea-pig pad. Of these, 12 reacted with both, two with C.A.M. but not with guinea-pig pad and one in the reverse way. This would suggest that the non-specific factors were not identical in the guinea-pig and C.A.M. The 12 sera reacting with both were then used to determine the number of non-specific reactions obtained with preparations of normal

TABLE I

PERCENTAGE OF NON-SPECIFIC FIXATION WITH CONTROL ANTIGENS FROM UNINFECTED MATERIAL

Method of Preparing Antigen	No. of Sera Tested	No. Reacting Non-specifically	Percentage Non-specific Reactions
C.A.M. (Hayward, 1949)	102	23	22·6
Guinea-pig pad (Bedson and Bland, 1929) ..	49	7	14·3
Allantoic-amniotic fluid (Gajdusek, Robbins and Robbins, 1952) ..	109	0	0

TABLE II

COMPARISON OF METHODS OF PREPARING ANTIGENS: I, NON-SPECIFIC FIXATION WITH PREPARATIONS OF NORMAL TISSUE AND SELECTED SERA*; II, POTENCY OF SPECIFIC ANTIGEN PREPARED FROM INFECTED TISSUE

Method of Preparation	I		II
	No. of Sera Tested	No. of Sera Reacting Non-specifically	Potency of Herpes Simplex C.F. Antigen Prepared from Infected Tissues
C.A.M.:			
Dudgeon (1950) ..	12	10	Good
Hayward (1949)+ chloroform	6	2	Fourfold loss in antigenicity after $CHCl_3$ treatment
Hayward (1949)+ freezing	6	6	Good
Casals (1949) ..	5	0	Poor
Guinea-pig pad:			
Antigen+$CHCl_3$ ..	—	—	Fourfold loss in antigenicity after $CHCl_3$ treatment
Mouse brain antigen:			
Casals (1949) ..	2	0	Poor
Casals and Palacios (1941)	—	—	Nil

* Sera reacting non-specifically with Hayward's C.A.M. antigen (1949).

tissues made by other methods and their modifications as used for making specific antigens. The results are given in Table II. As can be seen, the only two methods which reduced the non-specific reactions were treatment with chloroform and Casals' method, both of which, however, gave a poor yield of specific antigen.

Dudgeon (1950) has recommended inactivating human sera at 62° C. for 15 minutes to nullify the non-specific complement-fixing factors in them. We have found that such treatment, although reducing the non-specific effect, also reduced in some sera specific antibody. With four anti-herpetic sera, two showed a drop in titre. Three other sera containing antibodies to lymphocytic chorio-meningitis virus also showed a lower titre when heated similarly. If the sera were diluted in saline before heating, this fall in antibody did not occur (Felix and Olitzki, 1929), but neither was the non-specific factor reduced, for it also was protected by the diluent. The effect of heating on these four sera which contained relatively labile antibodies to these two viruses is given in Table III.

TABLE III

EFFECT OF HEAT ON ANTIBODIES TO HERPES SIMPLEX AND LYMPHOCYTIC CHORIO-MENINGITIS VIRUSES

Serum	Test Virus	Serum Heated	C.F. Titre after			
			56° C. for 30 min.	56° C. for 60 min.	56° C. for 30 min. + 62° C. for 15 min.	62° C. for 15 min.
D	Herpes	Neat	1/20	1/20	1/5	1/5
L	,,	,,	1/40	1/40	1/20	1/20
C	Lympho-cytic chorio-meningitis	,,	1/16	1/16	—	1/8
C	,,	Diluted	1/16	1/16	1/16	1/16
R	,,	Neat	1/8	1/8	1/2	1/4

Non-specific reactions were much more frequent during the spring and early summer than at other times of the year, an observation also noted with lymphocytic chorio-meningitis antigen prepared from guinea-pig spleen.

The preparation of antigen control also caused some difficulty. Six controls prepared in the same way from normal chorio-allantoic membranes were titrated against a serum containing non-specific factors at high dilution. The antigens were tested at dilutions of 1 in 10 and 1 in 20; two of them gave no reaction with serum diluted 1 in 5; a third reacted with serum at 1 in 10 and 1 in 5 respectively for the two antigen dilutions; a fourth with serum at 1 in 20 and 1 in 5; a fifth with serum at 1 in 5 for the stronger dilution of antigen control; and the sixth with serum at more than 1 in 160 with both dilutions of control antigen. In order to use the normal C.A.M. extract as a control, it was necessary to perform a chessboard titration with it against a serum reacting non-specifically just as was done with the viral antigens. The allantoic and amniotic fluids did not react in this way. Although often these infected fluids could not be used as specific antigens at as high a dilution as antigens prepared from the C.A.M. or guinea-pig pad and required more eggs for the same number of tests, they were the antigens of choice.

Of a hundred human sera tested, 80 contained neutralizing antibody and all of these except three had complement-fixing antibody to the allantoic or amniotic antigens. These three sera presumably had enough antibody for detection by the more sensitive neutralization test but not enough to fix the amount of complement used in the complement-fixation test. Hayward (1949) and Holzel *et al.* (1953) found an occasional serum which contained neutralizing antibodies but which did not fix complement with either allantoic or chorio-allantoic membrane antigens. No sera fixed complement specifically with these antigens which did not contain neutralizing antibodies.

With allantoic-amniotic antigens titres of complement-fixing antibody in human sera varied from 1 in 2 to 1 in 32 in different sera. The work of Brown (1953) would indicate that the antibodies involved in the complement-fixation test with the chorio-allantoic membrane antigen were different from those reacting in the neutralization test. In the case of allantoic-amniotic antigens, the comparison between the titre of the neutralizing and complement-fixing antibodies in seven human sera would suggest that they were the same, the ratio of the one to the other being constant within the error of the neutralization test (see Table IV). This point has yet to be clarified, but if it is correct

TABLE IV

RELATIONSHIP OF TITRES OF NEUTRALIZING AND COMPLEMENT-FIXING ANTIBODIES IN HUMAN SERA

Serum	Reciprocals of		Ratio (a)/(b)
	(a) PD_{50}* Titre	(b) Complement-fixing Titre	
1	118	60	1·97
2	33	40	0·8
3	142	80	1·8
4	124	80	1·7
5	37	40	0·9
6	36	30	1·2
7	37	40	0·9

* PD_{50} = Dilution of serum neutralizing 50% of the expected number of pocks produced by the test virus.

it would place the complement-fixing antigens of herpes simplex virus in a similar category to those of mumps (Henle, Harris, and Henle, 1948).

Using allantoic-amniotic antigens, we were unable to demonstrate complement-fixing antibodies in guinea-pigs which, after repeated injection with herpes virus, had produced neutralizing antibody. After the corneal scarification of rabbits, both complement-fixing and neutralizing antibodies appeared simultaneously, reaching a maximum in five to six weeks and remaining at that level for at least three months.

Although the allantoic-amniotic antigens are to be preferred for the diagnosis of herpes simplex infection by complement fixation, certain points in their preparation and use must be noted.

(1) An egg-adapted strain is necessary to produce a high enough titre of virus to give complement fixation. In our hands the McC strain was satisfactory while the E.W.H., which had only been through 10 egg passages, was not, a titre of 2×10^7 pocks per ml. being necessary for minimal fixation.

(2) The dose used must be such as to ensure survival of embryos for at least four days after yolk sac inoculation, as those dying earlier than this rarely yielded enough virus for satisfactory antigen production. Eggs should be candled twice daily and harvested just before or immediately after death of the embryo. Fluid contaminated by yolk should be discarded.

(3) To find the optimal dilution for use in diagnostic tests, the chessboard titration must be performed. The easier method of testing falling dilutions of antigen against a constant serum dilution is not satisfactory, as serum with a low titre of complement-fixing antibody may be missed if the latter method is used.

(4) If the standard serum used as the control shows a titre less than the usual value, the test should be repeated.

(5) As 3% of sera with neutralizing antibody were negative by complement fixation, it might be advisable to ensure that samples were not procomplementary, and to perform a neutralization test in certain of these cases.

(6) Excess complement in the tests should be avoided, as 2 M.H.D.s appeared to be the largest amount that could be used satisfactorily in our complement-fixing system.

Summary

Different methods recommended for the preparation of complement-fixing antigen with herpes simplex virus were compared. The most satisfactory antigen was infected allantoic or amniotic fluid of developing chick embryos, although the yield was not as great as that obtained from other sources. The absence of non-specific reactions with these fluids weighed heavily in their favour. The effect of heat on antibodies and the relation between neutralizing and complement-fixing antibodies in herpes simplex infections were discussed briefly. Certain precautions in the preparation of allantoic-amniotic antigens were noted.

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THE DETERMINATION OF ANTISTREPTOLYSIN

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Hodge and Swift (1933) found that the haemolytic activity of a given batch of streptolysin O did not give a satisfactory index of its ability to combine with antibody. For the purpose of standardizing the antistreptolysin titration (A.S.T.), therefore, it is necessary to determine the combining power of the lysin by reference to antistreptolysin of known concentration. The use of standardized whole serum from patients with raised antistreptolysin titres has not proved satisfactory in this laboratory for this purpose, owing to the fluctuations in titre which such serum has been found to show over a period of time. The following is an account of a method designed for routine use which employs standard antistreptolysin globulin derived from pooled serum.

Materials

Preparation of Streptococcal Haemolysin.—The medium is a modification of that originally described by Todd and Hewitt (1932) and further modified by Hodge and Swift (1933).

Lean beef, freed from fat, is minced and added to tap water in the proportion of 500 g. to 1 litre of water. After infusion overnight at 4° C. the temperature is carefully raised to 85° C. and maintained at this level for one hour. Filtration through muslin and finally glass-wool yields a clear golden-yellow broth. To this is added proteose peptone (Difco) in the proportion of 20 g. per litre, and normal sodium hydroxide solution, 30 ml. per litre. The broth is reheated to 85° C. and held at this temperature for a further half-hour, after which time the precipitated phosphates are removed by filtration through Chardin paper. Sterilization is effected by autoclaving at 15 lb. pressure for 20 minutes. The final *p*H should be between 7.2 and 7.6.

The broth is enriched and buffered with the following solution :

Dextrose	3 g.
$NaHCO_3$	2 g.
NaCl	2 g.
$Na_2HPO_4.12H_2O$	2 g.
Aqua dest.	50 ml.

This solution must not be heated, and is sterilised by filtration through a bacteriological grade sintered glass filter. For use the buffer solution is added in the proportion of 1 part to 19 parts of broth.

The organism employed for the production of lysin is the Richards strain of *Str. pyogenes* Group A Type 3, and is obtainable from the National Collection of Type Cultures, Colindale (N.C.T.C. 8191).

In order to obtain maximum growth attention is drawn to the following practical points concerning the actual cultivation of the streptococcus. It is essential to confirm the purity of the culture used for inoculating the broth by preliminary subculture, as contaminating organisms frequently elaborate haemolysins, the presence of which would interfere with the specificity of the reaction. For the same reason it is advisable to check the sterility of the broth by incubating it at 37° C. for 24 hours before inoculation. Approximately 10 ml. of an actively growing (eight hour) culture is added to 1½ litres of broth whose temperature has been previously raised to 37° C. The optimum incubation time is 18 hours, and if this is exceeded the lysin titre tends to fall. It has been found convenient to carry out the incubation in a water-bath. After the specified time has elapsed the broth is centrifuged at about 2,000 r.p.m. to remove the bulk of the organisms, and the supernatant filtered through a bacteriological grade sintered glass filter. Sterility is maintained by the addition of sodium ethyl mercurithiosalicylate ("merthiolate," B.D.H.), 1 : 10,000.

In view of the variation in the haemolytic activity of streptolysin during storage, fresh lysin is not used until at least one week after its preparation.

Stored at 4° C. streptolysin O has retained its activity for over 12 months, and it has not been found necessary to reduce it in bulk as has been advocated by other workers.

Buffered Saline Solution.—The activity of streptolysin O is maximum at *p*H 6.5, and the isotonic saline used in all titrations is accordingly buffered at this *p*H using the following phosphate solution as recommended by Herbert and Todd (1941).*

$Na_2HPO_4.12H_2O$	18.11 g.
$NaH_2PO_4.2H_2O$	16.76 g.

These ingredients are dissolved in distilled water with the aid of gentle heat and made up to 1 litre.

* This paper contains a mi print, as a result of which the ratio of the phosphate salts has been reversed. The formula here described has been corrected accordingly.

For use 1 volume of the buffer solution is added to 1 volume of 0.158 M sodium chloride solution in distilled water. All subsequent references to "saline" in this paper are to this mixture.

Red Cell Suspension.—Fresh rabbit blood, collected in 3.8% sodium citrate solution, is "washed" three times by centrifuging with saline and finally made up to a 5% suspension.

Standard Antistreptolysin Globulin. — In this laboratory the test was standardized against a sample of antistreptolysin globulin from Dr. B. F. Massell.

Subsequently the preparation of standard globulin derived from patients' serum has been carried out according to the following method, which was described by Kendall (1937).

One hundred millilitres of undiluted pooled serum from patients found to have a raised A.S.T. is added to 50 ml. of saturated ammonium sulphate solution, and the precipitated euglobulin recovered by centrifugation. It is dissolved in 50 ml. of distilled water and reprecipitated with 25 ml. of saturated ammonium sulphate solution, after which the precipitate is finally dissolved in the minimum volume of distilled water necessary to effect complete solution. For the purpose of the work described here it is not necessary to repeat the precipitation five times as recommended by Kendall. The dissolved euglobulin fraction, after the addition of a drop of chloroform, is dialysed against normal saline until sulphate is undetectable (with barium chloride solution) in the saline. Continued dialysis using distilled water results in precipitation of the water-insoluble euglobulin which is removed from the solution by centrifugation and discarded. After the addition of "merthiolate" (1:10,000) the solution is filtered through a bacteriological grade sintered glass filter.

It is essential that all stages in the preparation of globulin solutions involving the use of high salt concentrations should be carried out in a cold-room (4° C.), but from the dialysis stage room temperature is permissible.

Prepared in this way batches of antistreptolysin globulin have maintained a constant titre, when stored at ordinary refrigerator temperature (4° C.), for at least 18 months.

Method

Principle.—The haemolytic activity of the reduced lysin against rabbit red cells is determined in the presence of 1 unit of antistreptolysin globulin. The patient's serum is then titrated for its haemolysis-inhibiting power against the amount of lysin which just fails to produce haemolysis in the presence of 1 unit of standard globulin, the reciprocal of this dilution of the serum giving the number of units of antistreptolysin per millilitre.

It will be appreciated that, as the test is carried out using a standardized amount of lysin which gives no haemolysis in the presence of 1 unit of antistreptolysin, the production of any degree of lysis in a mixture which includes (diluted) serum indicates that the serum contains less than 1 unit. Accordingly, actual measurement of the degree of haemolysis is unnecessary, and, furthermore, the number of red cells added need not be accurately determined. It has been found that this system is capable of giving reproducible results over long periods without showing any deviation from the original standard, and, furthermore, in view of the stability of the lysin it is not necessary to repeat the titration before each batch of tests but only at intervals of at least a month.

Reduction of Lysin.—Thioglycollic acid (thiol-acetic acid, B.D.H., $CH_2SH.COOH$) has been found to be a convenient reducing agent, and is used in the following way:

In a small conical flask 0.3 ml. thioglycollic acid is placed and 5.7 ml. distilled water. A few drops of B.D.H. "universal indicator" are added, and the solution neutralized with 60% sodium hydroxide solution to a pale straw colour (*p*H 6.0), and made up to a total volume of 15 ml. with (buffered) saline. This constitutes the reducing agent. Four parts of lysin are treated with one part of this solution at least 15 minutes before the lysin is required. Prepared in this way the reduced lysin is stable for two or three days, although it is preferable to prepare a fresh batch each day. Other workers use various reducing agents, e.g., sodium hydrosulphite, cysteine, sodium nitrite. The above is recommended for its consistent effect and ease of use.

Standardization of Lysin.—Having obtained the standard globulin of known combining activity in terms of units of antistreptolysin the first step is to determine the degree to which the lysin has to be diluted in order that 2 volumes of globulin solution diluted to contain 1 unit of antistreptolysin globulin per ml. just inhibits the haemolytic effect of 1 volume of the diluted lysin.

An example of a lysin titration will make this clear:

Reduced lysin (ml.) ..	1·0	1·0	1·0	1·0	1·0	1·0
Saline	1·0	1·2	1·4	1·6	1·8	2·0
Take 0·25 ml. of each dilution						
Diluted lysin	0·25	0·25	0·25	0·25	0·25	0·25
Standard globulin containing 1 unit/ml.	0·5	0·5	0·5	0·5	0·5	0·5
Water-bath at 37° C. for 15 min.						
5% cell suspension ..	0·25	0·25	0·25	0·25	0·25	0·25
Water-bath at 37° C. for 60 min.						
Degree of haemolysis ..	+	+	±	−	−	−

Thus, at a dilution of lysin 1.0:1.6, haemolysis is inhibited by 1 unit of antistreptolysin. It will be noted that the time allowed for the combination of lysin with its antibody is 15 minutes at 37° C. and 60 minutes for the haemolytic reaction.

For each serum to be tested 1 ml. of diluted lysin is required in the first instance; therefore, in the example above, for 50 tests, 20 ml. of reduced lysin is added to 32 ml. of saline.

While variations in the potency of different batches of lysin may be encountered, by adherence to the method of preparation described above, titres ranging from 1 in 1.6 to 1 in 3.6 can be consistently obtained.

Dilution of Patient's Serum.—Each serum, after inactivation at 56° C. for half an hour, is tested in varying dilutions for its inhibition of streptolysin diluted according to the titration value. This is carried out in two stages, the first of which is designed to show the range within which the titre lies, the second to determine more accurately the end-point of those sera found to have an A.S.T. of 200 units/ml. or more.

The scheme of serum dilution to be described limits the possible number of readings per test to seven, thus enabling as many as 70 or 80 sera to be conveniently examined in one batch.

Stage I.—The following dilutions of serum are prepared in four tubes :

	Dilution
0·25 ml. serum + 4·75 ml. saline	1 : 20
1 ml. of 1 : 20 dilution + 1·5 ml. saline	1 : 50
0·5 ml. of 1 : 20 dilution + 4·5 ml. saline..	1 : 200
1 ml. of 1 : 200 dilution + 1·5 ml. saline	1 : 500

It has been found expedient, when large numbers of tests are being carried out, to use an automatic pipetting device adjusted to deliver a constant volume of 1.5 ml. for the saline. Thus, the first and third tubes in the above dilution series receive 3 such volumes and the second and fourth 1 volume. The additional 0.25 ml. is then added to the first tube with an ordinary pipette.

Having prepared these dilutions of the sera, the following volumes are taken into 3 in. × ½ in. tubes :

Tube		Final Dilution
1	0·5 ml. of 1 : 50 dilution	1 : 50
2	0·2 ,, ,, 1 : 50 ,, + 0·3 ml. saline ..	1 : 125
3	0·5 ,, ,, 1 : 200 ,, ...	1 : 200
4	0·5 ,, ,, 1 : 500 ,, ...	1 : 500

To each tube is added 0.25 ml. of the lysin diluted according to its titration result, and after being shaken the tubes are left in a water-bath at 37° C. for 15 minutes.

After this time 0.25 ml. of 5% rabbit cell suspension is added to all tubes, which are shaken and returned to the water-bath for a further hour. It is desirable to shake the tubes again 15 minutes after the beginning of this stage.

When the tubes are finally removed from the water-bath they are left to stand at room temperature for about an hour in order to complete the sedimentation of unlysed cells, thus facilitating the examination of the supernatant. If this precaution is not observed inaccurate readings may result. Each tube is then inspected for haemolysis, and, as discussed above, the slightest degree detectable is regarded as significant.

Result	A.S.T.
(a) Haemolysis in all tubes	Less than 50 units
(b) ,, ,, tubes 2, 3, and 4	50 units
(c) ,, ,, ,, 3, 4	125 units
(d) Haemolysis in tube 4	200–500 units
(e) No haemolysis	500 units or more

Of these five categories, sera which fall into (d) and (e) are further titrated to determine their end-point more accurately.

Stage II : Category (d) Sera.—Of 1:200 dilution, 0.3 ml. + 0.2 ml. of saline is titrated, giving a final dilution of 1:333.

Category (e) Sera	Final Dilution
0·3 ml. of 1 : 500 dilution + 0·2 ml. of saline	1 : 833
0·2 ,, ,, 1 : 500 ,, + 0·3 ,, ,, ,,	1 : 1,250
0·1 ,, ,, 1 : 500 ,, + 0·4 ,, ,, ,,	1 : 2,500

Having prepared these extended dilutions the test is carried out as before, adding first lysin, incubating, and then cell suspension, finally reading in the same way. The A.S.T. is taken as the reciprocal of the dilution in the last tube showing no haemolysis.

Titres below 200 units per ml. are regarded as being within normal limits, and those of 200 units and above as being raised.

It should be noted that the titres resulting from this scheme of dilution are equally spaced logarithmically, with the exception of the first and last values, viz. :

Titre	Log.
50	1·7
125	2·1
200	2·3
333	2·5
500	2·7
833	2·9
1,250	3·1
2,500	3·4

Results are therefore suitable for expression as a geometric mean according to the usual formula :

$$\text{Geometric mean} = \text{Antilog} \frac{\Sigma(f.\log x)}{n}$$

The application of this statistical method as a means of controlling the standardization of the test has proved useful.

Comment

This method has been designed to enable batches of 70 or 80 specimens to be titrated completely in one day, and it is felt that the range of values

chosen in the dilution series meets the clinical requirements in this Unit. The scheme may be modified to meet any required range.

Summary

A method suitable for the routine determination of the serum antistreptolysin O level is described.

Based on inhibition by the antibody of haemolysis in a system comprising streptococcal lysin and red blood cells, the test is standardized by reference to a globulin solution of known antistreptolysin content.

I am indebted to Dr. E. J. Holborow not only for his constant encouragement in the preparation of this paper but also for his generosity in allowing me to describe the serum dilution system which he devised. My thanks are also due to Dr. L. E. Glynn for his helpful criticism.

Dr. Benedict F. Massell, The House of the Good Samaritan, 25, Binney Street, Boston, Mass., U.S.A., kindly supplied the sample of antistreptolysin globulin.

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A COMPARISON OF BLOOD AND EGG MEDIA FOR THE RAPID ISOLATION OF *MYCOBACTERIUM TUBERCULOSIS*

BY

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The rapid isolation of *Mycobacterium tuberculosis* (Maccabe and Gould, 1954) on a culture medium consisting of human blood, saponin, and agar has been described. It was stated that on this medium growth of *M. tuberculosis* was more rapid than on Löwenstein-Jensen medium, and that the medium was superior to Löwenstein-Jensen.

In an attempt to confirm this finding saponated blood agar was prepared here, and a small series of microscopically positive sputa was inoculated on to this medium in parallel with a laboratory-prepared Löwenstein-Jensen medium.

Subcultures of *M. tuberculosis* isolated from sputa were also compared on the two media.

Culture Media

Saponated Blood Agar.—Prepared as originally described: 5 ml. of 10% saponin is added to 95 ml. of citrated Group O human blood, the mixture heated to 55° C. and added to an equal amount of 4% agar solution at 45° C. Quantities, each of 10 ml., are sloped in sterilized "universal" containers.

Two varieties of saponated blood agar were prepared, one using saponin, B.D.H., the other using saponin, white, B.D.H.

Löwenstein-Jensen Medium (Mackie and McCartney, 1948).—The medium was modified by the addition of 0.5% of dextrose to the mineral salt solution and by inspissation once only at 75°–80° C. for 30 minutes; 10 ml. is placed in sterilized "universal" containers.

Tests

In the first series of cultures for comparison of saponated blood agar (S.B.A.) and Löwenstein-Jensen medium (L.-J.) the S.B.A. contained the brown saponin.

Thirty microscopically positive sputa, concentrated by Petroff's method, were inoculated simultaneously on to two slopes each of S.B.A. and L.-J. and incubated at 37° C. for two weeks.

A second series was later begun in which the S.B.A. contained the white saponin. In this series 24 microscopically positive sputa were similarly inoculated on to S.B.A. and L.-J. slopes and incubated for two weeks.

Subcultures from positive primary cultures were also set up on both media.

Results

First Series.—It was found that growth of *M. tuberculosis* was supported by the S.B.A. on primary culture, but neither early appearance of colonies nor luxuriance of growth could compare favourably with that of the L.-J. medium.

Only five of the 30 sputa yielded growth on S.B.A. in two weeks as against 24 on L.-J. medium.

There was also a high rate of contamination on S.B.A.: 12 of the 30 sputa rapidly became overgrown and useless compared with five contaminated cultures on L.-J. medium.

The possibility of the addition of penicillin to the S.B.A. to reduce the amount of contamination was considered, but was not attempted in either series.

Subcultures from 12 positive primary cultures on L.-J. medium were set up in parallel on S.B.A. and L.-J. media. Obvious growth appeared on each type of medium in an average of eight days, the amount of growth being similar at first, but later more luxuriant on L.-J. medium after further incubation to two or three weeks.

Second Series.—The results from primary cultures on the second type of S.B.A. (white saponin) were much better than those on the first type.

Twenty-one of the 24 sputa yielded growth on both S.B.A. and L.-J. media, in 16 cases obvious growth occurring at the same time on both types of medium.

Of the remaining eight sputa, four yielded growth one to three days earlier on L.-J. medium, and in one case only growth appeared earlier on S.B.A. than on L.-J. medium. Three specimens yielded contaminants only.

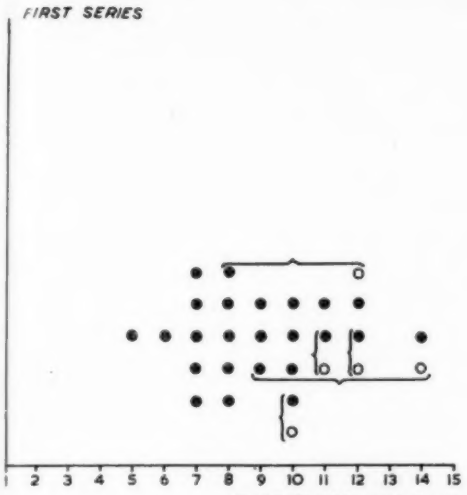
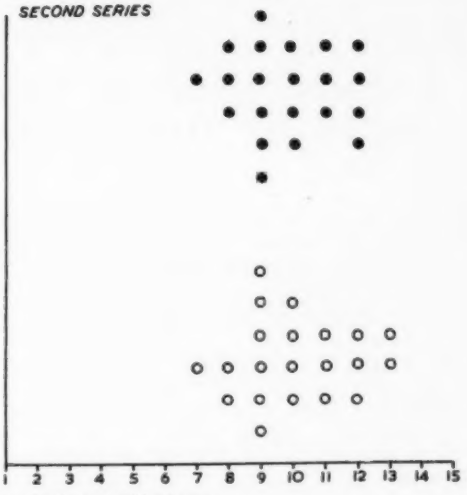


FIG. 1.—Diagram comparing the time of the first appearance of a typical surface growth of *M. tuberculosis* in primary culture (first series).



●=Modified Löwenstein-Jensen medium. ○=Saponated blood agar.

FIG. 2.—Diagram comparing the time of the first appearance of a typical surface growth of *M. tuberculosis* in primary culture (second series).

Subcultures, as in the first series, showed close similarity in the time of the first appearance of definite growth and in the amount of that growth.

Discussion

Although the number of sputa in both series is small, it would suggest that rapid primary growth of *M. tuberculosis* on saponated blood agar is not always readily producible in routine clinical laboratory work.

The superiority of white saponin over the ordinary commercial grade of saponin is indicated by a comparison of the two series.

One serious disadvantage of saponated blood agar was the high rate of contamination. The degree of contamination was frequently much worse on S.B.A., the whole surface of the slope being covered with bacterial growth and ruined in two or three days. Contaminating organisms on L.-J. usually spread upwards from the base of the slope and sometimes enabled colonies of *M. tuberculosis* to be isolated from the top of the slope. It is probable that the addition of penicillin, as used in penicillin-glycerol-blood agar (Tarshis, 1953), would reduce the number of contaminated slopes.

A further point in favour of the L.-J. medium was the ease with which developing colonies of *M. tuberculosis* could be distinguished from the original inoculum and from the majority of contaminants. On S.B.A. medium, the debris of the original inoculum tended to mask small colonies which at first could not be differentiated from the dark background.

The average time for the appearance of typical surface growth on modified Löwenstein-Jensen medium was 9.3 days and on saponated blood agar of the second series was 10 days, a difference of no significance; but in all the cultures, growth on Löwenstein-Jensen was more luxuriant and more easily identified in the earlier stages.

An interesting observation was the early appearance of typical developing cord-like colonies in the liquid at the base of the L.-J. slope (condensation water and primary inoculum). These have appeared as early as five days after inoculation, and can be seen readily using the $\frac{1}{3}$ in. objective in a Ziehl-Neelsen-stained film.

Summary

The growth of tubercle bacilli from microscopically positive sputa was compared on saponated blood agar using two types of saponin, and a modified Löwenstein-Jensen medium.

It was found that saponin (white) yielded a better blood agar medium than the brown saponin, but that both varieties of the medium were inferior to a modified Löwenstein-Jensen medium for primary isolation of *M. tuberculosis*.

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A HISTOCHEMICAL PROPERTY OF THE OXYPHIL CELLS OF THE HUMAN PARATHYROID GLANDS

BY

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(RECEIVED FOR PUBLICATION MAY 16, 1955)

It is the purpose of this communication to report a histochemical property of the "oxyphil" (so designated throughout this paper) or acidophil cells in human parathyroid glands, to re-examine the widely held view that such cells are merely involution forms of the principal cell, and to review the literature concerning their significance.

Material and Methods

Parathyroid glands were removed from routine necropsy cases, being mainly elderly subjects of both sexes. Gelatine-embedded blocks of formalin-fixed and post-chromed tissue were prepared and stained by Baker's (1946) acid-haematin technique for the histochemical detection of phopholipids. Near-by sections (although not strictly serial) were stained by haematoxylin and eosin. In each case a second parathyroid gland was treated as for Baker's pyridine-extraction-acid-haematin test. It would have been preferable to bisect a single gland and treat one half by the first part and the other by the second part of Baker's test, but, so far, glands of sufficient size have not been available.

No attempt was made to make a quantitative study of the number of cells rich in phospholipid granules in each parathyroid gland examined.

Brief Case Reports and Histological Observations

In elderly subjects oxyphil cells are more numerous and are often grouped together to form small nodules. This has fortunately been the case in several necropsy specimens, and it is proposed to select two of these for special study.

As full morphological descriptions of the oxyphil cells have already been given by numerous workers, in particular Welsh (1898) and Gilmour (1939), it only remains to describe their appearances in sections stained by Baker's histochemical technique, a positive result with which has not previously been reported.

Case 1.—This was a male, aged 72 years, who developed uraemia and died in 1953 two months after total cystectomy for a carcinoma of the urinary bladder.

In one parathyroid of this case there is a small, discrete nodule (Fig. 1), approximately ½ mm. in diameter, composed entirely of relatively large cells whose cytoplasm contains granules showing varying degrees of eosinophilia.

The pattern of pale and dark oxyphil cells within this nodule is identical with that depicted in Gilmour's (1939) colour plate (his Fig. 1); in addition, the histological appearances of each of these two types of oxyphil cell in this nodule conform to Gilmour's description of them. That this nodule is composed of oxyphil cells there can thus be no reasonable doubt. A near-by section (Fig. 2) taken from the same "run" of five or so frozen sections, and stained by Baker's acid-haematin test, that is, the first part of his test, shows many polygonal cells containing numerous fine black granules evenly dispersed throughout the cytoplasm of each cell. Some cells, often of crescentic shape with concave edges, are so heavily filled with granules that only in the thinnest parts of the sections can individual granules be discerned (Fig. 3). In sections approximately 7 or 8 μ thick, as in Fig. 3, these cells appear as dense black masses, but examination of such cells in thinner sections indicates that this picture is due to a dense accumulation of the same fine discrete particles (*vide infra*). Such cells clearly correspond to the dark oxyphil cells. There are other cells, also depicted in Fig. 3, with almost colourless cytoplasm in which acid-haematin-positive fine granules are present, but only sparsely so. Such cells correspond to pale oxyphil cells. In these latter cells one or sometimes two bodies, corresponding to the description given by Gilmour (1939) of intracellular accumulations of pigment, can be seen. With the first part of the acid-haematin test these bodies always give a positively reacting rim of variable thickness, and usually the centres also appear darkly stained except for one or two, or even more, almost colourless spaces which correspond to the vacuoles also described by Gilmour. There are also cells with varying numbers of evenly dispersed fine granules: such are clearly intermediate forms which are either accumulating or being depleted of their positively reacting contents.

In the acid-haematin preparations nuclear detail is not well shown, but in near-by sections of the same

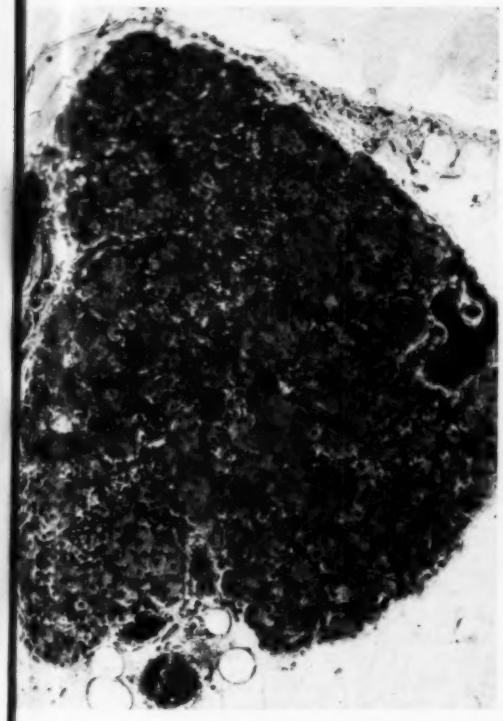


FIG. 1

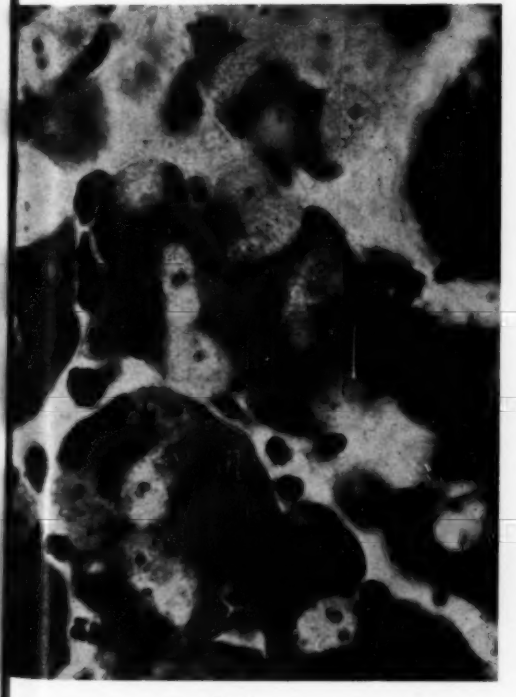


FIG. 2

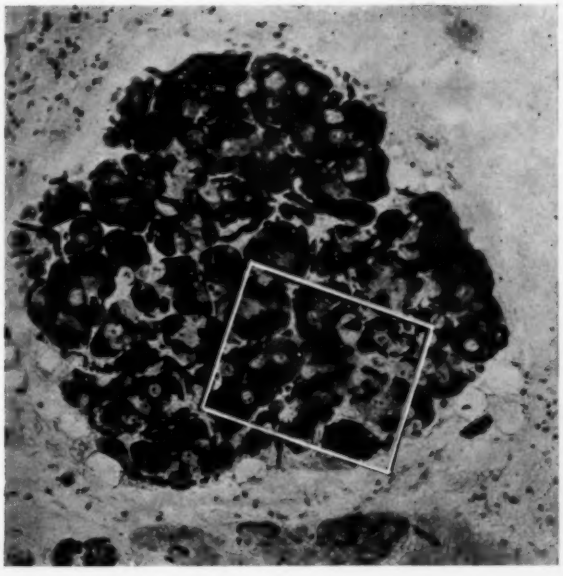


FIG. 3

FIG. 1.—Case 1: On the right-hand side is seen in cross-section a discrete nodule of oxyphil cells, whilst on the left is glandular tissue composed almost entirely of principal cells. Haematoxylin-and-eosin-stained section from same gelatine block as Fig. 2. × 200.

FIG. 2.—Case 1: On the right-hand side the nodule depicted in Fig. 1 is seen to consist of numerous dark oxyphil cells densely loaded with acid-haematin-positive granules, as well as cells either not so densely packed with or practically devoid of such granules. The mass of principal cells on the left is practically devoid of such granules: capillary channels containing heavily stained erythrocytes are clearly displayed. Baker's acid-haematin stain. × 200. (Although taken from the same block, sections depicted in this and Fig. 1 are not serial.)

FIG. 3.—Case 1: A higher magnification of the area outlined in Fig. 2. The concave borders of the densely granulated cells are well shown. × 900.

nodule stained by haematoxylin and eosin such detail is excellent. Welsh's (1898) description of the oxyphil cell nucleus cannot be bettered, viz. :

> "The nucleus is both relatively and absolutely smaller than the nucleus of the hyaline cell (he is here referring to what is now known as the 'principal' cell). In outline it [the nucleus of the oxyphil cell] is more nearly circular. Its chromatin is more densely arranged, so that no nuclear network can be detected, and the entire nuclear mass appears uniformly darkly stained."

When sections of this nodule are only lightly stained with haematoxylin and eosin some detail is apparent even in the densest nuclei, and pyknotic nuclei are very rarely seen. It is important to emphasize this point, because I consider that I have not yet seen cytological evidence to suggest that foci of oxyphil cells such as are present in this case and in that to be described below are, in fact, zones of cells undergoing involution. There is, however, in this nodule, which appears to consist entirely of oxyphil cells, considerable variation in nuclear size and chromatin content, and although the average size is, as observed by Welsh, smaller than that of the principal cell nucleus, there are all gradations from larger vesicular nuclei to smaller ones with fairly densely aggregated chromatin, but still possessing a definite pattern with numerous fine more or less evenly knotted chromatin threads.

A prominent feature of this nodule is its degree of vascularity, equal to that seen in parts of the gland containing principal cells.

In conclusion, the general histological appearance of this nodule is that of a gland, separate from the rest of the parathyroid tissue and composed of cells of similar type but in various stages of secretory activity. The degree of vascularity is also strong histological evidence of secretory activity rather than of inactivity and involution.

Case 2.—This was a female, aged 70 years, who succumbed to widespread lymphosarcoma with lymphatic leukaemia in 1953.

In one parathyroid there is a discrete oval nodule 1.0 by 0.5 mm. in size (Fig. 4), which is composed entirely of oxyphil cells except for a very small focus of principal cells at one end, constituting less than one-fifth of the area of the nodule in the plane of the section depicted in Fig. 4. Although the haematoxylin-and-eosin-stained preparation shows some, but by no means marked, variation in the degree of eosinophilia of the cytoplasmic contents of these relatively large polygonal cells (Fig. 5), Baker's acid-haematin stain, applied to a section only a few microns deeper in the same gelatine block, shows cells which clearly correspond to pale and dark oxyphil cells respectively, as well as a considerable number of intermediate forms (Fig. 6). The dark, heavily granulated cells, like those described in Case 1, have concave borders, whereas the pale, less heavily granulated cells have not. Occasionally, when sections are examined under the 2 mm. objective, very small intracytoplasmic bodies with a dark rim but colourless centre can be seen in some of the sparsely granulated cells : these almost certainly correspond to the minute vesicles described by Gilmour (1939).

This nodule, composed predominantly of oxyphil cells, is very well endowed with capillaries, many of which are filled with erythrocytes (Fig. 5), the degree of vascularity being certainly equal to, if not actually exceeding, that of the rest of the gland. There is also no cytological evidence of cellular degeneration or involution of the nodule as a whole.

In neither of the above cases could sections treated by the pyridine-extraction-acid-haematin part of Baker's test be seen to contain cells rich in positively reacting granules, hence the oxyphil cells give a true positive reaction with this test.

Discussion

Since the oxyphil cells were first clearly described by Welsh (1898)* little attention has been paid to them, although their existence as a cell type separate from the more numerous principal cells has been generally accepted (Gilmour, 1939 ; Maximow and Bloom, 1948 ; Nonidez and Windle, 1953) ; nevertheless some workers (Kurokawa, 1925 ; Hunter and Turnbull, 1931) have maintained that they are derived from the principal cell.

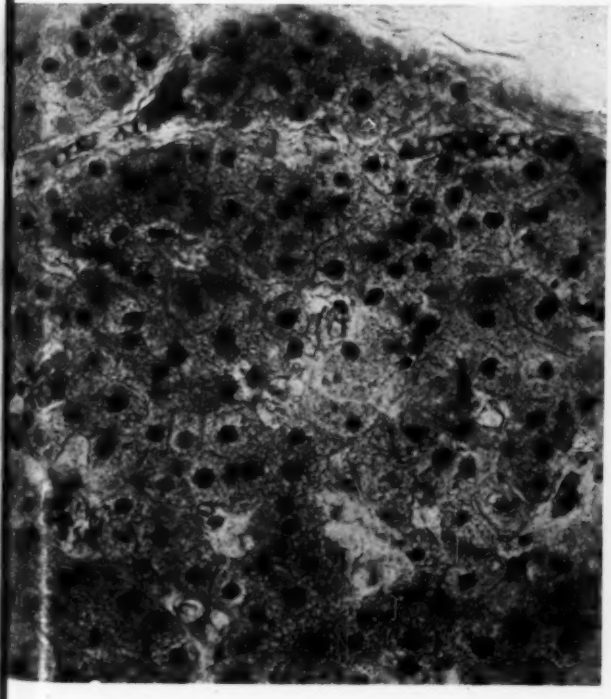
Oxyphil cells have not been described in domestic animals (Trautmann and Fiebiger, 1952) other than the ox (Levine, 1928). Smith and Copenhaver (1944) specifically stated that they do not occur in the dog, cat, or rat ; and Foster (1943, 1946) did not mention them in his studies on the mouse parathyroid. Levine recorded that oxyphil cells appear to be less frequent in steers than in cows, and noted that the isolated cells and clusters of cells in cows' glands have all the morphological and staining characteristics of the oxyphil cells in human material. He also considered that further evidence is found here for the contention that a physiological age factor operates in determining the presence of the oxyphil cells in these animals as it does in man.

There is also evidence to suggest that oxyphil cells are more numerous in women than in men. Gilmour (1939) observed that the number of such cells was comparatively very great in 22 out of 428 subjects which he studied : excluding cases of renal disease, the number was reduced to 15, all of whom were females. The ages of the 22 subjects ranged from 41 to 76 and 10 were over 60 years of age. He concluded that "there appears, therefore, to be a definite relationship between excep-

* Sandström (1880) was the first worker to mention these cells, although he did not give a very detailed description of them.

FIG. 4.—Case 2: A nodule composed predominantly of oxyphil cells except for a small, fairly well circumscribed nodule of principal cells, indicated by arrow. Haematoxylin and eosin. × 160.

FIG. 5.—Case 2: A higher magnification of part of the area depicted in Fig. 4, showing a predominance of pale oxyphil cells. Haematoxylin and eosin. × 900.



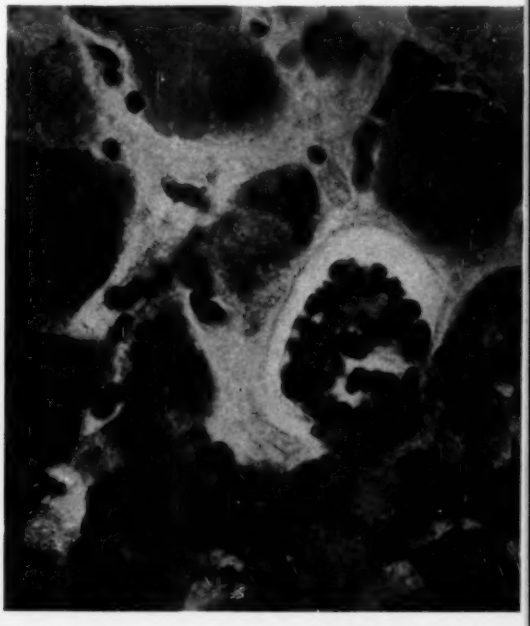


FIG. 6.—Case 2: A section from the same gelatine block as in Figs. 4 and 5 (although not actually a serial section) depicting mostly pale, but also a few densely stained dark, oxyphil cells. The considerable degree of vascularity is clearly apparent. Baker's acid-haematin stain. × 900.

tionally numerous oxyphil cells in the normal parathyroid and both age and sex."

It is interesting to note that, of the six cases of true oxyphil cell adenomata which the author has been able to collect from the literature, five have been in females.

Anderson (1953) also observed that these cells increase with age. As to the age at which they first appear there is some doubt: Erdheim (1901) considered about the tenth year, but Fischer (1911) saw them in a child aged 2 years, and Gilmour (1939) reported their presence in all subjects aged 3 years and over whom he examined.

Extensive histochemical studies of the parathyroid glands do not appear to have been undertaken. However, it is widely known that the principal cells are rich in glycogen, an observation first attributed to Petersen (1903). On the other hand, oxyphil cells are said to contain little or no glycogen (Gilmour, 1939; Stephenson and McNamara, 1948).

In 1946 Baker introduced a carefully standardized technique developed from the Smith–Dietrich test for the histochemical detection of lipins. In a later communication (1947) he claimed that this modification (the acid-haematin test), controlled by his pyridine-extraction test, allows histochemical recognition of phospholipids. This was confirmed by Cain (1947) while working in Baker's laboratory. Casselman (1952) applied this technique *in vitro* to certain synthetic enantiomeric α-phosphatidic acids, phospholipids, and related compounds, and to highly purified, natural lipin fractions, as well as to an additional 123 other substances: only phospholipids and certain phosphatidic acids give positive results.

Baker's histochemical test gives a positive reaction for phospholipids in the case of the pituitary acidophil cells in rats (Rennels, 1953), and also in the guinea-pig and man (unpublished work).

The resemblance between the pituitary acidophil cells and the parathyroid oxyphil cells was first emphasized as far back as 1898 by Welsh, who referred to it as "striking," and more recently by Maximow and Bloom (1948). It is now further enhanced by the fact that they both react similarly with Baker's test. This certainly does not imply exact chemical identity, but does suggest the possibility that the granules are probably composed, either in part or wholly, of substances belonging to a similar group of chemical compounds. (This observation may also prove of value in the chemical extraction and identification of the substances within these cells.)

When visual comparisons are made of the relative size of the positively reacting granules in the pituitary acidophil, parathyroid oxyphil, and argentaffin (Kultschitzky) cells in human material, it is apparent that in the former two they are of approximately the same size and considerably larger than in the last-mentioned. The fine granules of the argentaffin cell also give a positive reaction with Baker's stain (Christie, 1954).

As regards the function of the oxyphil cells nothing is known. Castleman and Mallory (1935) contended that ". . . it can fairly be said that histological evidence fails to support the concept of the elaboration of the parathyroid hormone by the oxyphil cells." Black and Ackerman (1950) and Nonidez and Windle (1953) also contended that the principal cells appear to be responsible for secreting the parathyroid hormone.

Some workers have considered that the oxyphil cells are likely to be involution forms—for example, Castleman and Mallory (1935) stated that "the frequency of these cells in tumours, even in young people, their absence in hyperplasias, their increase under normal conditions in old age, all point towards an involution phenomenon." All the same, they did admit that the possibility of another function, unconnected with calcium metabolism, could not be ruled out, although they found no evidence for it.

A detailed histological study of the parathyroid glands in both the cases reported above as well as in many other glands has failed to convince the writer that there is any evidence of a cytological nature to suggest that oxyphil cell collections (large or small) are foci of cell degeneration or involution; in particular, the marked degree of vascularity throughout such zones, with numerous open capillaries, is evidence to the contrary. Such vascularity has been observed in the case of other actively secreting endocrine organs as, for example, the islets of Langerhans and the pituitary gland.

Furthermore, Baker's histochemical technique provides yet additional evidence of similarity with the pituitary acidophil cells whose histological significance as an endocrine unit is beyond doubt.

Excluding neoplasms, there are four well recognized conditions in which hyperplastic parathyroid glands are encountered, namely, rickets (or osteomalacia), pregnancy, renal insufficiency of the type associated with phosphate retention, and calcium deprivation (Albright and Reifenstein, 1948). Parathyroid hyperplasia has also been found in association with extensive skeletal tumours such as multiple myeloma and metastatic carcinoma (Ben-Asher, 1939). The histology of the para-

thyroid glands in pregnancy has apparently not been studied in any detail.

Not all workers agree on the effects of renal disease on the number of oxyphil cells, that is, whether they increase or decrease. Fritz and Brines (1951) examined the parathyroids of 61 cases of bilateral renal disease; while finding a definite increase in total glandular weight as compared with 76 control cases without renal disease, they observed no increase in the number of oxyphil cells. On the other hand, Castleman and Mallory (1937) examined the glands in 29 cases of chronic renal insufficiency and found that the oxyphil cells are always greatly increased in number. In his monograph (1947) Gilmour dealt with the parathyroid glands in renal disease and referred to several cases in which oxyphil cells were numerous, being particularly so in two cases, females aged 40 and 60 years respectively. Black and Ackerman (1950) considered that oxyphil cell adenomata may appear in patients with long-standing renal disease, and reported two such cases. One of these was a female, aged 59 years, suffering from osteoporosis of the vertebrae, rheumatoid arthritis, hypertensive heart disease, and nephritis, who was submitted to operation for exploration of the parathyroids, all of which were enlarged to about twice their normal size with the exception of the left, which was considerably larger (3 by 3 by 1.3 cm.) and composed entirely of oxyphil cells. Following removal of this oxyphil cell adenoma there were no significant changes in the serum calcium, phosphorus, or alkaline phosphatase values — observations which they considered strong evidence that the oxyphil cell adenoma was not secreting parathormone.

The second relevant case described by Black and Ackerman (their Case 13) was a female aged 66 years, who gave a somewhat similar history and comparable biochemical findings as well as showing evidence of renal calculi and osteoporosis. At necropsy severe renal damage was found and death was attributed to this cause, but in addition a parathyroid oxyphil cell adenoma, measuring 4.3 by 2.2 by 1.9 cm., was found.

It now remains to discuss the rarely occurring tumours composed either entirely of, or with a predominance of, oxyphil cells. Norris (1947) collected 322 cases of parathyroid adenoma from the literature over 43 years (1903–45 inclusive): of these, only four were classified as oxyphil-cell adenomata (Hunter and Turnbull, 1931; Cope, 1944; McQuillan, 1938; Norris, 1946). Of these, only the latter two workers' case reports show convincing photomicrographic evidence of typical oxyphil cells, and neither case showed evidence of osteitis fibrosa or the other stigmata associated with principal cell tumours.

McQuillan's case—a solitary tumour measuring 9 by 6 cm. in a female aged 46 years—is particularly interesting in that the tumour appears to have arisen following childbirth and has since regularly become enlarged during the menses. Norris's case was a female, aged 23 years, who had a solitary oxyphil adenoma measuring 3.5 cm. in diameter. Keynes (1936) and Hadfield and Rogers (1932) have each reported typical oxyphil cell tumours, the former worker's case being a female aged 50 years, in which the tumour weighed 18 g.; and the latter's case, a female aged 58 years, in which the tumour weighed 52 g. Once again skeletal and other evidence of hyperparathyroidism was absent in each case.

There have been several reports of cases with pluriglandular adenomatosis involving the pituitary and parathyroid glands, and sometimes the pancreas and adrenals as well. The pituitary tumour has consisted of either chromophobe cells (Lloyd, 1929; Kalbfleisch, 1937; Underdahl, Woolner, and Black, 1953, Case 6; Marshall and Sloper, 1954) or acidophil cells (Cushing and Davidoff, 1927, Case 3). Of Cushing and Davidoff's (1927) four cases of acromegaly of differing clinical types, only Case 3, a male aged 34 years, described as "typical acromegaly of circa 15 years' duration," showed parathyroid hyperplasia, and then of slight degree—only two glands were identified, the larger being 9 mm. in diameter. Each gland contained small adenomata consisting predominantly of principal cells, although the larger did contain a small peripheral zone of oxyphil cells.

Likewise in Lloyd's (1929) case—a 22-year-old female showing, at necropsy, a large pituitary chromophobe adenoma, adenoma-like enlargements of the islands of Langerhans, parathyroid glands and adrenals—the principal cell predominated in two enlarged parathyroid glands (the third gland located was of normal size).

Of three cases of pluriglandular adenomatosis described by Kalbfleisch (1937), in the third, a male aged 23 years, there was, in addition to a malignant tumour of the anterior lobe of the pituitary gland and an adenoma of the pancreas, slight hyperplasia of the parathyroid glands, particularly the left lower one (found embedded in the thymus). The principal cell predominated in the hyperplastic glands, but a few oxyphil cells were also present in all four glands. (There was

clinical evidence of calcium deficiency in the skeleton.)

Also in Shelburne and McLaughlin's (1945) case —a seaman aged 26 years, with radiological and clinical evidence of a pituitary tumour and surgically removed solitary adenomata of both the pancreatic islets of Langerhans and parathyroid glands respectively—the predominant cell type in the latter, although not stated, was presumably the principal cell, as there was clinical evidence of hyperparathyroidism with a high serum calcium level and recurrent renal calculi.

Willis (1948) reported an interesting finding in an obese woman of 55 years who died the day after operation for multiple large uterine myomas. Necropsy disclosed a yellowish ovoid tumour measuring 2.5 by 1.5 cm. in the position of one of the inferior parathyroids. Microscopically it was a parathyroid adenoma, composed mainly of large, solid, liver-like oxyphil cells, but also with areas of small cuboidal cells in acinus-like clusters and of vacuolated clear cells. In addition, there was a meningioma, 4 cm. in diameter, in the cerebello-pontine angle and multiple cortical adenomata of both adrenal glands. (Willis remarked that the multiplicity of tumours in this case was noteworthy.)

Underdahl *et al.* (1953) reviewed the world literature relating to such multiple endocrine adenomata for the period 1903–51. They added seven cases of their own. One of these, a very obese male, aged 28 years, had a chromophobe-cell pituitary tumour, multiple parathyroid and islet-cell tumours, adenomatous nodules of adrenal cortices, and "diffuse polyposis of the gastric mucosa with giant rugae characteristic of Menetrier's disease." In the three enlarged parathyroids there were four small adenomata, one, of the order of 0.5 cm. in diameter as measured from the photograph, being composed of oxyphil cells. It is interesting to note that both Willis's and this case were reported as being obese, the latter (Underdahl's) being particularly so (307 lb.).

More recently Marshall and Sloper (1954) reported the case of a male aged 40 years with a chromophobe-cell pituitary adenoma, islet cell adenomata of the pancreas, multiple adrenal adenomata, and a small solitary parathyroid tumour (1.7 by 0.8 cm.) consisting predominantly of chief and water-clear cells except for one lobule composed entirely of oxyphil cells.

It thus appears that oxyphil-cell adenomata do occur but rarely, and are not associated with skeletal or other changes characteristic of hyperparathyroidism. Also when associated with other endocrine tumours they are usually very small and do not constitute the chief cell component of the enlarged organs, Willis's case being an exception.

Of the six cases of predominantly, or completely, oxyphil-cell adenomata unassociated with renal disease referred to above, five occurred in females, and one, that of Underdahl *et al.* (1953), in a male. The latter's oxyphil-cell tumour was, as mentioned above, only small (0.5 cm. in diameter) and associated with larger adenomata of other endocrine glands; consequently solitary tumours of moderate or large size involving these cells have all been in females.

As there is such a close histological similarity between the pituitary acidophil cells and the parathyroid oxyphil cells the possibility exists that they may have a similar function, and that adenomata of the latter might be responsible for a clinical syndrome resembling acromegaly either in its classical or "fugitive" form (Bailey and Cushing, 1928). However, there is no evidence from the above-mentioned case reports that such is the case: in fact the few case reports of solitary oxyphil cell tumours referred to earlier have been unassociated with any clinical syndrome. If such tumours are secreting an excess of a hormone, different from parathormone, which has definite clinical effects both when deficient and in excess, then it appears that such an increase has no adverse clinical effect. (Such is the case with an interstitial cell tumour of the testis in an adult male.)

The histochemical stain described above is considered a valuable aid in histological technique to distinguish more clearly between principal cells of various types on the one hand and true oxyphil cells on the other. It may help in the further study of tumours composed of Castleman and Mallory's (1935) "transition oxyphil" cells.

Summary

Oxyphil cells in human parathyroid glands give a positive reaction with Baker's acid-haematin histochemical test for phospholipids, thus revealing a further resemblance between these cells and the acidophil cells of the pituitary gland.

Histological evidence is adduced to indicate that there is no reason to believe that the oxyphil cells are degenerate or involution forms of the principal cell.

Various clinical conditions in which oxyphil cell hyperplasia has been reported are briefly referred to. There is no apparent association with acromegaly either in its classical form or Bailey and Cushing's (1928) "fugitive" types. When the

parathyroid is involved in pluriglandular adenomatosis, the lesion is predominantly a principal cell hyperplasia.

A search of the literature has indicated that the rare tumour composed predominantly of oxyphil cells occurs mainly in females. It does not give rise to the clinical manifestations of osteitis fibrosa.

It is considered that a complete reinvestigation of these cells in normal healthy subjects of both sexes of all ages, in various disease states, and especially in pregnancy (both normal and abnormal) should be undertaken. A constant search should also be made for further examples of oxyphil-cell adenomata in life as well as at necropsy. In the latter instance a thorough search should be made for tumours or other lesions elsewhere, particularly in the light of the findings reported by Willis (1948)

I wish to thank Mr. Alan Hunt and Dr. P. Thompson Hancock, of the Royal Marsden Hospital, London, for permission to use material from their Cases 1 and 2 respectively. My thanks are due to Mr. F. E. Speed, of the Chester Beatty Research Institute, London, for the photomicrographs.

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HIBERNOMA (GRANULAR CELL LIPOMA)

BY

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Hibernomas are peculiar and apparently rare benign fatty tumours believed to originate from human brown fatty tissue homologous to the hibernating glandular fatty tissues found in animals. Brines and Johnson (1949) were able to find only nine apparently authentic cases in the literature to which they added one of their own. Six more have since been recorded, all from American sources. We wish to report two further cases.

Case Reports

Case 1.—A woman aged 47 was admitted to hospital for investigation of a lump in the right buttock. She was not able to say with certainty how long this had been present, but thought that the right buttock had always been larger than the left. She had attended for treatment because of a dull aching pain in her leg which had been present for about a year. There was no relevant previous medical or family history.

On examination she was found to have a soft, mobile subcutaneous swelling some 10 cm. in diameter in the upper and outer quadrant of the right buttock, which was considered clinically to be a lipoma. There were no other abnormal clinical findings.

FIG. 1.—Hibernoma in Case 1.

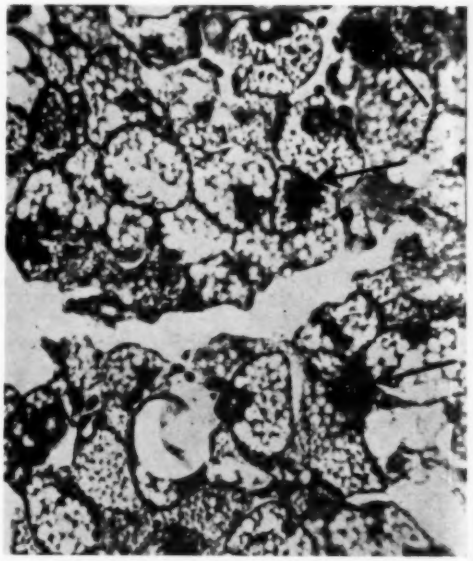

FIG. 2.—Histological section of hibernoma (Case 1) showing typical tumour cells and intracytoplasmic bodies (arrows). (Haematoxylin and eosin, × 320.)

At operation a fairly well circumscribed tumour was present in the subcutaneous soft tissues of the buttock. A complete resection was performed, although some difficulty was experienced because of the intimate connexion of part of the tumour with the tendon of the gluteus maximus.

Convalescence was uneventful, and there has been no sign of recurrence for three months following operation.

The tumour was rounded, measuring 9 by 7 by 5 cm. and weighing 165 g. (Fig. 1). It was encapsulated, firm, and presented a uniform lobulated tan-brown appearance on section. Histologically fibrous tissue septa traversed the tumour, dividing it into variously sized lobules. These were composed of closely packed large (20–100 μ) round, oval, or polygonal cells showing well-defined cell boundaries. The nuclei were

central or eccentric but not peripheral, and showed a finely distributed chromatin network. One to three prominent nucleoli were present. The cytoplasm was of a granular, eosinophilic nature with few or many rounded loculi. These granules and vacuoles stained positively with Sudan IV.

In haematoxylin-and-eosin stained sections, round or oval pink-staining intracytoplasmic bodies were found (Fig. 2). These stained a lighter pink than the ordinary granules and were quite distinct from them. They stained positively with Sudan black and the periodic-acid-Schiff stain, and were not acid-fast. They did not stain with Sudan IV. They were considered to be of a phospholipid nature. Stains for free iron were negative.

Case 2.—A miner aged 32 was admitted to hospital complaining of a lump on the left side of the back. This had been noticed for the first time by his wife eight weeks previously and was slowly getting larger.

On examination there was a smooth, soft, mobile subcutaneous swelling to the left of the scapula measuring 5 by 7 cm. One small axillary gland was palpable. The clinical diagnosis was "lipoma."

At operation a well circumscribed tumour was removed completely from the deep surface of the left trapezius. It was uniformly lobulated and of tan-brown colour on section. The histological appearances and staining reactions were identical to those of the first case, except that the pink-staining intracytoplasmic bodies were not present (Fig. 3).

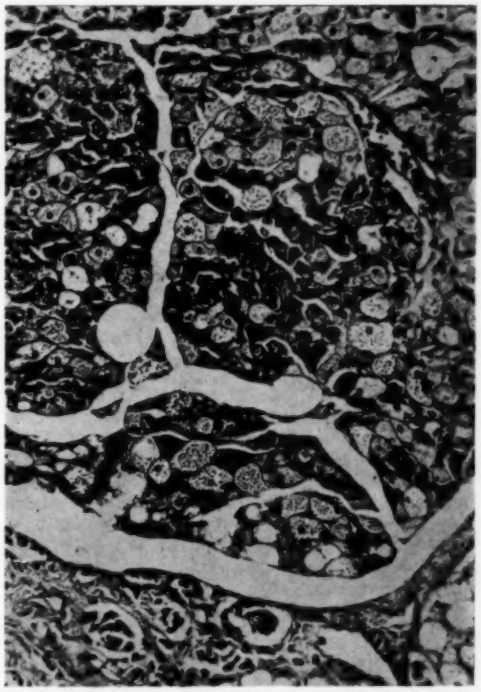

FIG. 3.—Histological section of hibernoma (Case 2) showing interlobular connective tissue stroma and typical granular and multilocular fat cells. (Haematoxylin and eosin, × 140.)

Discussion

Merkel (1906) described a case of a pseudolipoma in the breast of a woman of 26, which is probably the first report of a hibernoma. He was impressed by its unusual histological appearance and likened it to a fatty adenoma of sebaceous gland. Gery, in a discussion on a case reported by Bonnel (1914), introduced the term hibernoma because of its histological similarity to the hibernating gland in animals. Hibernal masses of lobular fatty tissue formed of so-called brown fat have been described in a wide variety of hibernating animals and non-hibernating rodents (Auerbach, 1902; Rasmussen, 1923). Interscapular, mediastinal, cervical, lumbar, inguinal, and gluteal masses have been recorded. In man the interscapular gland is a paired, lobulated irregular fatty organ situated on the shoulder and extending into the neck (Bonnot, 1908). It is limited externally by the deep fascia and internally by the prevertebral fascia. It is believed to represent the human homologue of hibernating fatty tissue in animals (Hatai, 1902; Bonnot, 1908) although Rasmussen was not convinced on this point. The features common to these fatty collections in normal animals and man and the hibernoma are the brown colour, lobular structure, and the multilocular appearance of the fat cells.

Brines and Johnson (1949) in their review of the literature on hibernoma were able to find only nine probable cases previously described and seven further doubtful ones. They added one case of their own, a girl aged 18 with a typical hibernoma in the axillary region. Since that time, six cases, all from American sources, have been reported. Two of these were intrathoracic (Kittle, Boley, and Schafer, 1950; Gross and Wood, 1953), one posterior cervical (Simon, 1950), one axillary (Gross and Wood, 1953), one popliteal (Sieber and Heller, 1952), and one in the subcutaneous tissue of the back (Sutherland, Callahan, and Campbell, 1952).

Macroscopically, these tumours are encapsulated and firm. On section they are of an unusual tan-brown colour, which has been likened to the appearance of boiled muscle (Brines and Johnson, 1949). The histological appearances are unique and quite unlike ordinary lipomas or liposarcomas. Connective tissue septa traverse

the tumour, dividing it into well-marked lobules. These are composed of closely packed large cells (20–100 μ in diameter), round, oval, or polygonal in shape, possessing well-defined cell boundaries. The nuclei are usually centrally situated, showing a loose chromatin network and one to three prominent nucleoli. Mitoses were not seen in either of our cases. The cytoplasm is granular and eosinophilic with multilocular fat spaces. All gradations are seen between cells with much granular cytoplasm and few fat loculi and those with many fat globules and few, if any, granules. In our Case 1, in addition to these cytoplasmic granules, round or oval eosinophilic intracytoplasma bodies up to 15 μ in diameter were present. These stained positively with Sudan black and periodic-acid-Schiff stain, and were considered to be of a phospholipid nature. We have not seen any record of such a finding in previously reported cases.

Other authors (Brines and Johnson, 1949; Simon, 1950) have observed in these tumours normal adult fat cells with peripherally placed signet ring nuclei, but we have been unable to confirm these findings. Unilocular round spaces were present amongst otherwise typical multilocular fat cells, but they did not show eccentrically placed nuclei and did not stain positively with fat stains. They appeared to be spaces resulting from the degeneration of granular fat cells, with absorption of the previously contained fat. There has been much controversy concerning the histogenesis of yellow fat, and its origin still remains uncertain. Flemming (1879) considered that it developed from ordinary connective tissue cells, whereas Toldt (1888) advanced the conception of specific mesenchymal precursors.

Stout (1944) has shown from studies *in vitro* that the lipoblast, a special form of connective tissue cell distinct from the fibroblast, is the precursor of adult fat cells and derived, like it, from primitive mesenchymal cells. Whether the lipoblast or some other specialized cell is the precursor of multilocular brown fat cells is not known. Transmutation between these cells and unilocular fat cells would appear from the evidence available to be unlikely to occur. Hammar (1895) was unable to produce unilocular fat cells from the multilocular type in the rat by excessive feeding, and Rasmussen found no intermediate forms, although both types of cell did occur together. However, Metzner (1890) and Cramer (1920) suggested that the granules are transformed into vacuoles which eventually coalesce and form the single large fat globule of the normal adult fat cell.

Hibernomas appear to be specific oncological entities, probably arising from granular, multilocular fat cells or their precursors. It is interesting that no true mixed forms showing lipomatous and hibernomatous features have been reported. Hibernomas must be distinguished from atypical lipomas composed of incompletely differentiated fat cells and from granular myoblastomas which may give a very similar picture in haematoxylin-eosin preparations but will be recognized by the use of fat stains.

We have found no definite instance of a malignant form of this tumour. However, Lever (1954) depicts a photomicrograph of a malignant hibernoma although there is no detailed reference in the text to this case.

Chemical analyses of hibernomas have been performed by Brines and Johnson (1949), Kittle *et al.* (1950), and Gross and Wood (1953). The results of these workers show that these tumours contain a similar total fat content to ordinary lipomas, although possessing in some instances a higher unsaponifiable fraction.

Although the term hibernoma has been in use for some 40 years, it is suggested that the designation "granular cell lipoma" would be more appropriate until their histogenesis is finally established.

Summary

Two further hibernomas are described.

The literature is briefly reviewed and the relationship of these tumours to hibernating fatty tissue is discussed. It is suggested that the term "granular cell lipoma" would be more appropriate until their histogenesis is more definitely established.

We wish to thank the late Mr. F. H. Scotson and Mr. R. O. Davidson for permission to publish details of their cases, and Dr. J. Davson for his helpful criticism and advice.

Miss J. L. Perry provided the photomicrographs.

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A CASE OF DISSECTING ANEURYSM IN A CHILD

BY

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A case is reported of sudden death in a girl aged 10 years due to the rupture of a dissecting aneurysm of the ascending aorta. Cases of dissecting aneurysm have been very rarely reported in children, and the occasional occurrence of such cases has added interest because it strengthens the view that dissecting aneurysms in younger patients are of congenital origin.

Case Report

The patient, a girl aged 10 years, was said to have always been delicate, subject to colds and the like. At the age of 3 years she had meningitis which left her with a squint, later corrected at operation. On the afternoon of her death she played a game of "tig" with her friends, running about continuously for half an hour. She then suddenly ran into her home and collapsed semi-conscious on to the floor. By the time the family doctor arrived she was making a good recovery but complaining of pains in the chest and abdomen. Physical examination was negative except for signs of a mild bronchitis, so the patient was put to bed, the doctor saying that he would visit her again the next day. During the evening the child was described as very lively, but before she went to sleep beside her mother she again complained of pain, this time in the chest and neck. Waking in the morning, the mother found her daughter dead beside her.

Necropsy was performed at the request of the coroner. Nothing abnormal was noted at the external examination. The cause of death was found to be a haemopericardium which had originated in a small external tear in a dissecting aneurysm limited to the ascending aorta. On opening the heart and aorta, a horizontal split was seen in the wall of the aorta situated 2¼ cm. above the aortic valves (Fig. 1). Dissection had occurred into the outer third of the media (Fig. 2). There was some dilatation of the ascending aorta, the lumen being 2.8 cm. in diameter. At a point just beyond the origin of the subclavian artery the arch of the aorta showed a degree of constriction, the lumen being reduced to 9 mm. in diameter to open out again so that in the descending thoracic aorta the diameter was 1.5 cm. This minor degree of coarctation was not associated with any development of collateral circulation. The heart weighed 200 g. The foramen ovale was patent (8 mm. in diameter). There was also an inconspicuous cord-like thickening running just below, and parallel to, the free margin of the aortic valves (Fig. 1). The only other abnormality noted at necropsy concerned the kidneys. The right kidney was hypoplastic and lay on the pelvic wall in relation to the external iliac artery. The left kidney was in the normal position and showed a compensatory hyperplasia.

Sections taken from the wall of the aorta showed an extensive degeneration of the media which had its greatest development in the region within 3 cm. of the aortic valves. Here the wall of the aorta contained clefts and cyst-like spaces. The degeneration particularly affected the elastic tissue (Figs. 3 and 4). Appropriately stained sections showed that in the first 3 cm. of the aorta elastic fibres were virtually confined to the outer third of the media, and that here they were separated and broken up, and in places interrupted by the cyst-like spaces. In the distal part of the ascending aorta and in the arch, the elastic tissue extended throughout the wall, but showed separation and fragmentation of the fibres. In the sections stained by haematoxylin and eosin (Fig. 5) relatively normal smooth muscle could be seen, the individual fibres, in places, being separated by empty spaces staining faintly blue. There was a complete absence of any signs of inflammatory reaction. Vasa vasorum were frequent, but only in the first 3 cm. of the aorta. In places these vessels, having entered the outer third of the media, showed local dilatations. Sections were also stained by Southgate's mucicarmin and by the P.A.S. techniques because a number of the reports on medial degeneration of the aorta describe a mucinous degeneration. Mucin was not demonstrated.

The pulmonary artery in the region just above the valves showed, in a minor degree, similar degenerative changes to those observed in the aorta, the elastic fibres being separated and fragmentated (Fig. 6). Sections from the aortic valves showed that the cord-like thickening referred to above was composed of oedematous fibrous tissue.

Discussion

It is now well recognized that dissecting aneurysm sometimes occurs in the young. Schnitker and Bayer (1944), considering 580 cases of dissecting aneurysm gathered from the litera-

FIG. 1.—Aortic valves and beginning of the ascending aorta showing the horizontal split in the wall of the aorta and the cord-like thickening running below the free margin of the valves (approx. natural size).

ture, found that in 141 cases (24%) the patients were less than 40 years of age. Gore (1953) collected 32 cases of dissecting aneurysm in patients under 40 years of age from the records of the U.S. Armed Forces Institute of Pathology. The condition, however, has rarely been reported in children: Schnitker and Bayer's survey included only four cases in the age group 1–10 years.

Brief details are given below of cases that have been reported in children and adolescents. The details for the cases reported before 1906 are those quoted in the reviews of the literature given in the papers referred to above, and in papers by Bronson and Sutherland (1918) and by Klotz and Simpson (1932).

Bronson and Sutherland (1918) described what must be one of the youngest cases. The patient, a boy, showed clinical signs of aneurysm at the age of 4½ years. Rupture of a dissecting aneurysm into the wall of a previously existing saccular aneurysm of the arch of the aorta occurred at the age of 6 years. A minor degree of coarctation was present, and there were other developmental defects the significance of which will be discussed below. Cases of dissecting aneurysm of the arch of the aorta in two boys both aged 8 years and a girl aged 9 years were reported by C. Rokitansky (1838), K. Rokitansky (1852), and Oppenheimer (1905). In K. Rokitansky's case there were developmental defects: congenital narrowing of

FIG. 2.—Section of the wall of the aorta showing the beginning of the dissecting aneurysm into the outer third of the media (×20 Weigert's elastic stain and neutral red).

the aorta, a ventricular septal defect, and deformity of the pulmonary artery.

Cases occurring in slightly older patients include those reported by Horder (1907) in a boy aged 11 years, dissection occurring into a previously formed saccular aneurysm of the aortic arch ; by Wasastjerna (1903) in a boy aged 13 years with an extreme degree of coarctation ; and by Gardner, Galbraith, and Hardwick (1939). In Gardner's case, a boy of 15, the aneurysm was described as the largest dissecting aneurysm that had ever been reported, the dissection extending into the main arterial trunks of the limbs. There was also a dissecting aneurysm of the pulmonary artery, which is of interest in view of the finding of medial degeneration of the pulmonary artery in the present case.

It is generally agreed that the degeneration of the media is the cause of nearly all dissecting aneurysms, including those occurring in the young. Of the cases quoted above, medial degeneration was mentioned in the case reports by Bronson and Sutherland, Oppenheimer, and Gardner *et al.* The severe medial degeneration observed in the present case has been described. Degeneration particularly affected the elastic fibres, and it is of interest that Gore found that in young patients degeneration of the media was primarily that of elastic tissue whereas in older patients it affected the muscle.

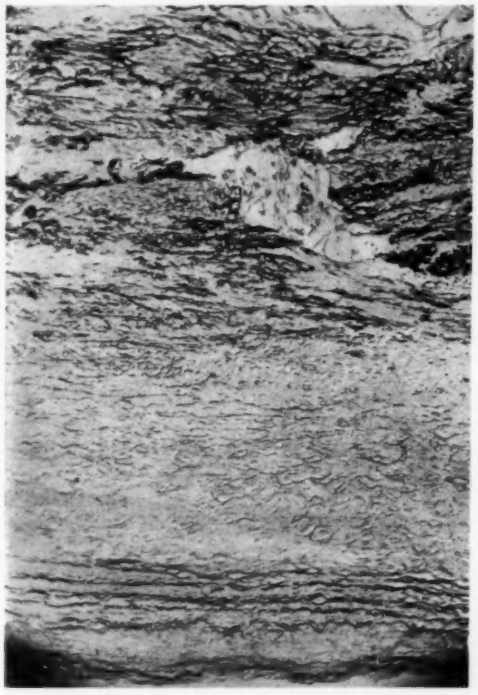

FIG. 3.—The wall of the aorta 2 cm. above the valves showing disappearance of elastic from the inner two-thirds of the media and fragmentation and separation of the fibres in the outer third (×75 Weigert's elastic stain and neutral red).

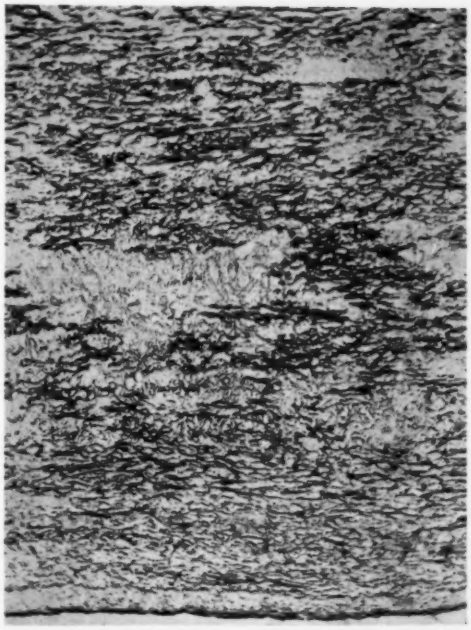

FIG. 4.—The wall of the aorta just distal to the split. Severe degeneration of the elastic tissue but less so than in Fig. 3. (×75 Weigert's elastic stain and neutral red).

The aetiology of medial degeneration may be reviewed briefly. It seems probable that it differs, when it occurs in later life, the degeneration then being a part of the changes of age, from when it occurs in younger patients (Sloper and Storey, 1953). In young patients medial degeneration is probably of congenital origin. Evidence for this view is afforded by the occasional occurrence of dissecting aneurysm in children and the association with developmental defects. The frequent association of medial degeneration when it occurs in young patients with developmental defects of the cardiovascular system is the subject of comment by Schnitker and Bayer and by Gore. By far the commonest association is with coarctation. When this association occurs it would seem that the medial degeneration should be regarded as a

further developmental defect additional to the coarctation and not simply as secondary to the mechanical strain. Medial degeneration and rupture can occur when stenosis is slight and in some cases has occurred distal to the coarctation (Reifenstein, Levine, and Gross, 1947). Less frequently observed congenital defects included bicuspid aortic valves and a patent foramen ovale.

There is an important association between medial degeneration and the syndrome of developmental defects known as Marfan's syndrome. This syndrome includes long slender digits (arachnodactyly), looseness of the joints, and dislocation of the lenses of the eyes. The literature of the relatively few cases that have come to necropsy has been reviewed by Marvel and Genovese (1951) and Goyette and Palmer (1953); further cases not included in these reviews have been reported by Sloper and Storey (1953), Gore (1953), and Whittaker and Sheehan (1954). Out of a total of 40 cases, aneurysms due to medial degeneration were observed in 26, 18 being dissecting and eight fusiform. In Marfan's syndrome medial degeneration has usually not become manifest until early adult life; in all cases but one, rupture of an aneurysm did not take place until after the patients had reached 20 years of age. However, there was one case of ruptured aneurysm in a girl aged 14 years, and in two other cases in which death occurred in adult life clinical evidence of dilatation of the aorta was noted in childhood. It is also probable that Bronson and Sutherland's case of ruptured aneurysm at the early age of 6 years was an example of Marfan's syndrome. Arachnodactyly was not noted, but the report refers to looseness of the joints, chest deformities, a high arched palate, and an unusually shaped head with prominent ears—all defects that have been described in Marfan's syndrome.

Incomplete examples of the syndrome with lesions restricted to the cardiovascular system can occur, and in such cases the diagnosis can sometimes be made certain by study of the family tree (Whittaker and Sheehan, 1954). It has indeed been suggested that many of the cases of dissecting aneurysm that have occurred in young patients were incomplete examples of Marfan's syndrome. The presence of coarctation need not preclude such a classification, since in three cases coarctation has been found to be associated with Marfan's syndrome. It should be remembered, however, that

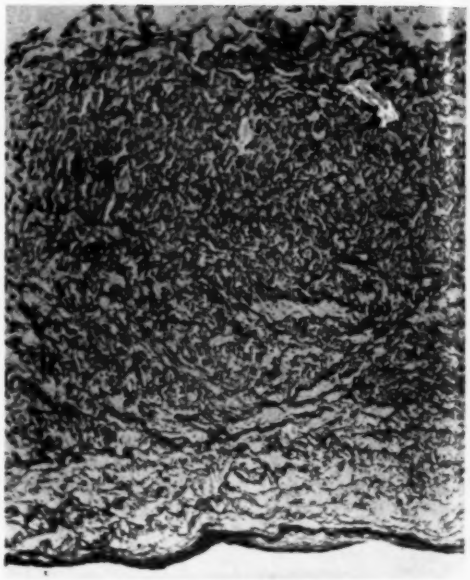

FIG. 6.—The wall of the pulmonary artery just above the valves. Separation and fragmentation of the elastic fibres (×75 Weigert's elastic stain and neutral red).

FIG. 5.—The wall of the aorta 2 cm. above the valves. Relatively normal smooth muscle fibres separated by empty spaces staining faintly blue. Local dilatations of vasa vasorum and an area of interstitial haemorrhage (×75 haematoxylin and eosin).

the literature concerned with aneurysm due to medial degeneration in patients under 40 years includes some 200 cases, of which only about 26 have been attributed with certainty to Marfan's syndrome.

In the present case there was good evidence that the medial degeneration was of congenital origin. The aneurysm ruptured at an early age and there were other congenital defects : a patent foramen ovale, a minor degree of coarctation, and a hypoplastic right kidney situated in the pelvis. The stigmata of Marfan's syndrome were not observed. With respect to the eyes, superficial examination was all that was permissible at the necropsy, but the eyes had been carefully examined by an ophthalmic surgeon at the time of the squint operation and on a subsequent occasion one year before her death. No abnormality beyond amblyopia due to the squint was noted. Such inquiries as were possible into the family history were negative, but because of the circumstances of sudden death it was not considered kind to press these inquiries very far. Slender evidence connecting the case with Marfan's syndrome is perhaps afforded by the presence of a patent foramen ovale, since this defect is relatively common in cases of Marfan's syndrome (seven out of 40 cases) whereas it has been discovered infrequently in other cases of dissecting aneurysm in the young (four out of 170 cases). Also, a cord-like thickening along the free margin of the aortic valves somewhat similar to that observed in the present case has been observed in cases of Marfan's syndrome (Sloper and Storey, 1953).

Summary

A case of dissecting aneurysm of the aorta due to medial degeneration is described in a girl aged 10 years. The rarity of dissecting aneurysms in children is stressed. The aetiology of medial degeneration in the young is briefly discussed with reference to its association with developmental defects, particularly with coarctation and with Marfan's syndrome.

My thanks are due to Dr. F. E. Eddison, H.M. Coroner for W. District, North Riding, Yorks, for permission to publish the case, to Dr. L. G. Boyd for clinical details, and to Mr. W. R. I. Hollingsworth for technical assistance.

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VARIATIONS IN THE REACTION BETWEEN THROMBIN AND FIBRINOGEN AND THEIR EFFECT ON THE PROTHROMBIN TIME

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The appearance of fibrin provides the end-point in every clotting test, and the duration of the thrombin-fibrinogen reaction is thus a component of any measurement of clotting time. While many tests are designed to study the early stages of clotting, in interpreting the results it is necessary to consider to what extent the observed differences may be due simply to differences in the "indicator reaction" between thrombin and fibrinogen. This precaution particularly applies to one-stage tests in which comparisons are made between different bloods.

The reaction between thrombin and fibrinogen may be separately investigated, and fortunately the measurement of the thrombin clotting time of citrated plasma (Biggs and Macfarlane, 1953) is one of the simplest of all clotting tests. This paper reports a case in which the one-stage prothrombin time was spuriously lengthened by an abnormally slow thrombin-fibrinogen reaction; tests were then made in four other cases to investigate the processes involved.

TABLE I

PROTHROMBIN TIME AND VARIOUS THROMBIN CLOTTING TIMES WITH PLASMA FIBRINOGEN CONCENTRATIONS

Test	Mean Clotting Times (sec.)		Patient's Plasma Fibrinogen Concentration (g.%)
	Patient	Control	
One-stage prothrombin time:			
30/5/50 ..	28·5*	26·2	
20/6/51 ..	16·2	12·1	
Thrombin clotting time:			
30/5/50 ..	20	13	0·35
2/6/50 ..	19·8	11·0	
11/8/50 ..	9·6	Control 1 6·1	0·30
		" 2 5·2	
20/6/51† ..	15·3	8·0	

* The clotting entries carrying a decimal place are the means of two or three replicate readings.

† In the thrombin clotting time tests of 20/6/51, the clot appeared to form more gradually in the patient's plasma than in the control plasma.

Case Presentation

E. M., a man aged 29, had a malabsorption syndrome with ascites and tetany. The whole blood calcium was 5 mg.%, plasma albumin 1.25 g.%, plasma globulin 1.72 g.%, blood urea nitrogen 20 mg.%, blood fatty acids 405 mg.%, packed cell volume 34%, and cephalin flocculation +++.

The one-stage prothrombin time with human brain reagent was prolonged. The thrombin clotting time was observed over a period of a year and was always greatly prolonged. These results are shown in Table I, with two readings of the plasma fibrinogen concentration. The first of these was made by the micro-Kjeldahl nitrogen method and the second by a clot-weight method (Ingram, 1952a): both are within normal limits (Ingram, 1952b).

The following experiments were made on June 20, 1951:

Equal Mixture of Control and Patient's Plasma.—The prothrombin time and thrombin clotting time were intermediate between those of the control and the patient alone (Table II).

Isolation of Fibrinogen.—Equal volumes of control and patient's plasma were precipitated at ¼-saturation with ammonium sulphate and the precipitates were taken up in 0.85% saline; the precipitation was once repeated and the solutions were dialysed for 20 hours against saline. It was noted that the patient's precipitates spun down more readily than those from the control plasma. Samples of these solutions were diluted 50:50 with veronal buffer *p*H 6.8 (Owren,

TABLE II

PROTHROMBIN TIME AND THROMBIN CLOTTING TIME IN PATIENT'S AND CONTROL PLASMAS AND IN EQUAL MIXTURES

Test Substance	One-stage Prothrombin Time (sec.)	Thrombin Clotting Time (sec.)		
		Thrombin 1	Thrombin 2	Thrombin 3
Patient's plasma	16	10	14	*ca* 22
Patient-control plasma mixture*	13·1	5·6	8·6	14·3
Control plasma*	12·1	4·8	7·1	11·0

* Each entry is the mean of three readings.

1949) and the thrombin clotting times repeated. The patient's fibrinogen clotted more slowly than that from the control (patient's mean, 7.5 sec.; control mean, 6.8 sec.; each is derived from five readings; for the difference between the means, $P<0.05$).

Tests on Defibrinated Plasma.—Equal volumes of plasma from control and patient were heated at 56° C. for 3 min. and cooled rapidly. The precipitated fibrinogens were removed by centrifugation and a solution of purified human fibrinogen (Lister Institute) was added equally to the supernatants, to a concentration of about 0.3 g.%. Samples of these plasma-fibrinogen mixtures were then mixed in equal proportions and the thrombin clotting time determined on all three mixtures. There was no significant difference between the mean results.

In conclusion, this patient appeared to have an abnormality of the fibrinogen which delayed the thrombin-fibrinogen reaction and thus apparently the one-stage prothrombin time.

Investigations in Other Cases

Effect on Prothrombin Time of More Rapid Thrombin-Fibrinogen Reaction. — The following experiments were made on citrated plasma taken from a patient showing a short thrombin clotting time (Table III).

TABLE III

MEAN THROMBIN CLOTTING TIMES IN PLASMA

Test Substance	Thrombin Clotting Time (sec.)			Fibrinogen Concentration in Citrated Plasma (g.%)
	Thrombin 1	Thrombin 2	Thrombin 3	
Patient's plasma ..	6·1*	24·2	70·8	0·39
Control ,, ..	6·4	26·1	74·4	0·32
Ratio: Patient's clotting time / Control clotting time† ..	0·95	0·93	0·95	

* Each mean is derived from four readings, and in each case the difference between patient and control means is significant ($P<0.05$).
† The ratios suggest that the magnitude of the effect is independent of the clotting time.

(1) Equal volumes of patient's and control plasma were precipitated at ⅓-saturation with ammonium sulphate. The precipitates were drained, dissolved in saline containing one-tenth volume of veronal buffer *p*H 6.8, and made up to the volumes of the original plasmas. The procedure was once repeated and the solutions dialysed overnight against the same solvent.

Plasma obtained from two other persons was pooled and defibrinated with a trace of thrombin.

Thrombin clotting times were then measured on each fibrinogen, both with and without the addition of defibrinated plasma, to confirm that the relative reactivity of the samples had not been greatly altered by the isolation (Table IV).

One-stage prothrombin times with human brain extract in four dilutions were then measured on each fibrinogen in clotting mixtures containing 0.1 ml. each

TABLE IV

MEAN THROMBIN CLOTTING TIMES IN SOLUTIONS OF FIBRINOGEN ISOLATED BY AMMONIUM SULPHATE PRECIPITATION

Test Substance	Thrombin Clotting Time (sec.)		
	Thrombin 1	Thrombin 2	Thrombin 3
Without defibrinated plasma:			
Patient's fibrinogen	5·9*	17·7	38·0
Control ,, ...	6·9	20·9	42·9
Ratio Patient's clotting time / Control clotting time ..	0·85†	0·85	0·89
With defibrinated plasma:			
Patient's fibrinogen	6·8	28·0	
Control ,, ...	7·7	32·2	
Ratio Patient's clotting time / Control clotting time ..	0·87†	0·90	

* Each mean is derived from three readings, and in each case the difference between patient and control means is significant ($P<0.01$).
† The ratios suggest that the magnitude of the effect is again independent of the clotting time.

of the defibrinated plasma, one of the fibrinogen solutions, brain extract, and M/40 calcium chloride. The results are given in Table V, which shows that the proportional difference between the clotting times from the two fibrinogens increased as the clotting times lengthened. Now, Table IV showed that the proportional differences between the thrombin clotting times of these fibrinogens did not increase as the clotting time lengthened, so Table V suggests that, as the prothrombin time lengthens, the time required for the interaction between thrombin and fibrinogen represents

TABLE V

MEAN PROTHROMBIN TIMES IN MIXTURES OF DEFIBRINATED PLASMA, FIBRINOGEN SOLUTION, BRAIN EXTRACT, AND M/40 CALCIUM CHLORIDE SOLUTION

Test Substance	1-Stage Prothrombin Time (sec.)			
	Brain 1/1	Brain 1/20	Brain 1/80	Brain 1/300
Patient's fibrinogen	12·2*	21·0	31·6	45·7
Control ,, ...	12·6	21·8	34·0	51·8
Ratio Patient's clotting time / Control clotting time	0·97	0·96	0·93	0·88

* Each mean is derived from three readings, and the increasing difference between the pairs of means, as shown by the falling ratios, is highly significant ($P<0.01$: tested on the interaction between brain concentrations and fibrinogens in an analysis of variance made on log. clotting times).

an increasing fraction of the interval elapsing between recalcification and the appearance of clot. A hypothetical example will make this clear.

Suppose, for instance, that the thrombin-fibrinogen reaction occupies one-tenth of the whole prothrombin time when this is 20 sec., and two-tenths when, by dilution of the brain reagent, the prothrombin time is increased to 50 sec.; then, in the one case the thrombin-fibrinogen reaction will constitute the final two seconds and in the other, the final 10 seconds. Suppose, further, that in two plasmas the thrombin-fibrinogen reactions proceed at differing rates in the

ratio 7 : 10. Other things being equal, the prothrombin times of these plasmas will follow the values given in Table VI. The differences shown in the tests will be relatively smaller with concentrated brain and relatively greater with dilute brain. If it is alternatively assumed that the thrombin-fibrinogen reaction occupies a constant fraction of the prothrombin time, this effect does not appear.

TABLE VI
HYPOTHETICAL EXAMPLE

Test Substance	1-Stage Prothrombin Time (sec.)	
	Concentrated Brain	Dilute Brain
"Fast" plasma ..	18+2×0·7=19·4	40+10×0·7=47
"Slow" "	18+2 =20·0	40+10 =50
Ratio "Fast" plasma clotting time / "Slow" plasma clotting time	0·97	0·94

(2) Two weeks later further samples of citrated blood were obtained from this patient and from a different control subject. Fibrinogen was isolated by the method of Jaques (1943), employing phosphate buffers at *p*H 6.6, and dialysed against saline overnight. The results were similar to those in the previous experiment : the mean thrombin clotting times of the patient and control fibrinogen solutions alone (each derived from four readings) were 7.9 sec. and 10.0 sec. respectively (ratio, 0.79) ; and the mean prothrombin times with diluted brain extract on defibrinated plasma were 36.6 sec. and 42.1 sec. respectively (each derived from four readings : ratio, 0.86). These and the previous findings were broadly in agreement.

In conclusion, the effect on the one-stage prothrombin time suggested that, as the clotting time was lengthened by dilution of the brain reagent, the thrombin-fibrinogen reaction came to occupy an increasing fraction of the interval recorded as the prothrombin time.

The Influence of Other Factors.—Citrated plasma was obtained from another patient showing a rapid thrombin clotting time relative to a normal control.

Effect of Differently Reacting Fibrinogens upon Earlier Processes Involved in Prothrombin Time.—Fibrinogens were isolated by Jaques's method from the patient's and control plasmas as before. Table VII gives the thrombin clotting times on the fibrinogen solutions alone and the prothrombin times with defibrinated plasma from another source, as in the previous experiments. The results are similar to those from the previous case.

TABLE VII
MEAN CLOTTING TIMES ON ISOLATED FIBRINOGENS

Test Substance	Thrombin Clotting Time (sec.) on Fibrinogen Alone	1-Stage Prothrombin Time (sec.) with Defibrinated Plasma Added*
Patient's fibrinogen	7·6 (3)†	47·6 (2)
Control " ..	9·5 (3)	50·7 (2)
Patient's clotting time / Control clotting time	0·81	0·94

* The brain extract was diluted.
† The number of readings contributing to each mean is shown in parenthesis.

These samples were then used to test the hypothesis that the "fast" fibrinogen sample contained an accelerator of thrombin generation, whose activity might explain the difference in the prothrombin times when the fibrinogens were tested with defibrinated plasma. Two-stage prothrombin time curves were obtained at 23° C. from mixtures containing 67% defibrinated plasma, 28% fibrinogen solution, and 1.7% molar calcium chloride solution. Duplicate curves were obtained from each fibrinogen with brain extract in final concentrations of 2.8% and of 0.28%. The mixtures clotted on the rising limbs of the curves at about 1 min. with the concentrated brain extract and at about 1.5 min. with the dilute extract. With each concentration of brain extract, the rising limbs of the four curves could be closely superimposed.

In conclusion, there was no evidence that the fibrinogen isolate contained an abnormal accelerator of thrombin generation.

Effect of Fibrinogen Concentration upon Differences in Thrombin Clotting Time.—Citrated plasma was obtained from a patient in whom the fibrinogen concentration was raised and the thrombin-fibrinogen reaction was delayed, relative to normal blood.

The fibrinogen concentrations were determined in the patient's (0.51 g.%) and the control (0.29 g.%) plasmas and a portion of the patient's plasma was diluted with saline to reduce the fibrinogen concentration to that of the control. Fibrinogen solutions were prepared from equal volumes of each sample by Jaques's method, as before. The fibrinogen concentration was determined on the three samples (patient, control, and diluted patient) and serial dilutions in saline were prepared from them. Two to four replicate readings of the thrombin clotting time

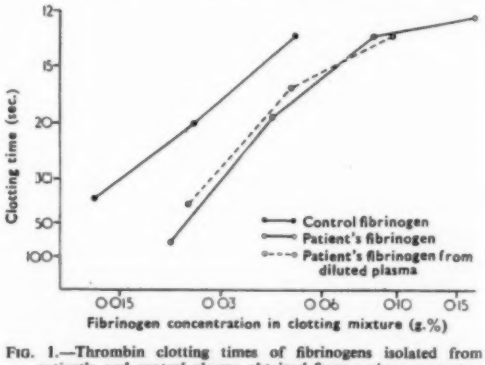

FIG. 1.—Thrombin clotting times of fibrinogens isolated from patient's and control plasma obtained from various concentrations of fibrinogen with one thrombin concentration. The ordinate is reciprocal, the abscissa logarithmic.

were then recorded from each dilution with a single thrombin concentration. The results are shown in Fig. 1, where the mean clotting times are plotted against the fibrinogen concentrations in the clotting reactions.

The graph shows three things. First, the relationship between fibrinogen concentration and thrombin clotting time (found by some observers and reviewed by Owren, 1947; confirmed by Shinowara, 1948) is clearly shown, with the optimum concentration being approached at about the normal plasma level. Second, the relative reactivity of the patient's and the control fibrinogen is independent of the concentration tested. Third, this relationship is also independent of the concentration of fibrinogen in the plasma samples from which the isolates were prepared.

In conclusion, the fibrinogen of this patient's plasma appeared to react with thrombin abnormally slowly. In a purified system, the effect was independent of the fibrinogen concentration and it was not a function of the difference between the concentrations of fibrinogen in the original plasmas.

Effect of Other Plasma Constituents upon Differences in Thrombin Clotting Time.—The thrombin clotting time was found to be more rapid in citrated plasma from another patient than in citrated plasma from either of two controls (patient, 9.0 sec.; control A, 9.7 sec.; control B, 10.8 sec.; each derived from four readings).

To determine whether the effect was related to differences between the fibrinogens or between other plasma constituents, equal samples of plasma from the patient and from control A (from whom the patient differed the less) were treated as follows:

One pair of samples was defibrinated with a trace of thrombin, and from another pair the fibrinogens were precipitated by ⅓-saturation with ammonium sulphate; these precipitates were drained with filter papers and taken up in distilled water to twice the volumes of the original plasma but were not dialysed. Equal mixtures of each plasma with each fibrinogen were prepared and thrombin clotting times obtained (Table VIII). In the results differences between the fibrinogens are apparent, but not between the plasmas.

TABLE VIII

MEAN THROMBIN CLOTTING TIMES (SEC., EACH DERIVED FROM FOUR READINGS) ON MIXTURES OF DEFIBRINATED PLASMA AND ISOLATED FIBRINOGEN FROM PATIENT AND CONTROL A

Plasma	Fibrinogen	
	Patient	Control A
Patient	11·2	14·6
Control A	11·6	14·7

The differences between the results from the two plasmas are not significant; but between the fibrinogens, $P<0.01$.

In conclusion, the rapid thrombin clotting time of this patient's plasma was a property of the fibrinogen and not of other plasma constituents.

Discussion

When blood clots spontaneously there is a latent period of some minutes before thrombin appears in the system, but thereafter the concentration rises sharply to about 10 units/ml.; the liberation of thrombin with brain extract follows a similar course, but the latent interval is much reduced (Macfarlane and Biggs, 1953). It is commonly observed in two-stage tests (as in the experiment reported above) that clotting occurs at a fairly low thrombin concentration but while the concentration of thrombin is still rising rapidly, so that under physiological conditions the rate of the thrombin-fibrinogen reaction may be partly determined by the rate of liberation of thrombin.

The rate of thrombin liberation was found to be reduced by dilution of the brain extract in two-stage tests on defibrinated plasma (Ingram, 1955). This reduction was comparable to the low rate of liberation of thrombin observed in thrombocytopenic and haemophilic blood (Macfarlane and Biggs, 1953). The supposition of a prolonged thrombin clotting time under such conditions agrees well with the results of the prothrombin time experiment in the second case studied.

In the thrombin clotting time test the concentration of thrombin is brought almost instantaneously to the test level, so that the conditions do not closely simulate the physiological clotting of fibrinogen. A rising concentration of thrombin would be difficult to reproduce in a simple test, but the ordinary method of measuring thrombin clotting time seems, from the above observations, to reflect at least some features of the normal process.

The biochemistry of the reaction between thrombin and fibrinogen has been extensively studied (Laki, 1953; Lóránd, 1954; Bailey and Bettelheim, 1955), and the experimental error of the measurement of thrombin clotting time is among the lowest of "one-stage" clotting tests (Ingram and Armitage, 1952; further details of experimental error are given in the Appendix). The reaction is believed to occupy the final two to five seconds in the clotting of normal blood in a glass tube (Biggs, 1955). Proceeding at this rapidity the reaction is unlikely to be affected by variations in "progressive antithrombin," and this has been confirmed by Lyttleton (1949).

Clinical Use of the Test.—It is convenient to mix 0.1 ml. of citrated plasma and 0.1 ml. of saline and then to time the reaction after adding 0.1 ml. of thrombin solution diluted so that the resulting clotting time is about 10–20 sec. These quantities

are economical in reagents and provide the same concentration of plasma as in the one-stage prothrombin time test.

The reaction between thrombin and fibrinogen is affected in various ways by ionized calcium (Robbins, 1944; Laki and Lóránd, 1948; Burstein, 1951; Ferry, Miller, and Shulman, 1951; Rosenfeld and Jánszky, 1952; Burstein and Guinand, 1954), so that for clinical investigation the reaction should be measured in recalcified plasma; this is conveniently done either by replacing the saline with M/40 calcium chloride solution or by adding to the diluted thrombin one-fortieth of its volume of molar calcium chloride solution.

Thrombin may be prepared by the method of Douglas and Biggs (1953). Prepared thrombin is available from various sources (e.g., the bovine thrombin, "topical," of Parke, Davis & Co.). In a comparative study of the thrombin clotting times with human and bovine thrombins, five out of six human plasmas reacted somewhat differently to the two thrombins, and it is therefore thought that human thrombin should be used for clinical investigations.

The thrombin clotting time might advantageously be measured more often in clinical work than current publications suggest to be the case. The test is affected by heparin, but may be returned to normal by adding toluidine blue (Biggs and Macfarlane, 1953). This therefore provides a simple indication of heparinaemia. The test becomes progressively more sensitive to heparin as the thrombin concentration is reduced (Biggs, 1955). It is also possible that clinical abnormalities of blood coagulation may be associated with disturbances of the thrombin-fibrinogen reaction, but little evidence has yet been presented.

Summary

A case is described in which a lengthening of the prothrombin time seemed to be due to an abnormally slow reaction between thrombin and fibrinogen.

Investigations in other patients suggested that, as the prothrombin time lengthened, the thrombin-fibrinogen reaction occupied an increasing fraction of the interval between recalcification and the appearance of the clot; and that differences observed between the thrombin clotting times in normal and abnormal plasmas depended upon a property of the fibrinogens and were independent of fibrinogen concentration, thrombin concentration, or other plasma constituents.

The measurement of the thrombin clotting time is a simple test and might advantageously be used more often. Technical details are discussed.

I am indebted to Dr. P. Armitage, of the Medical Research Council Statistical Research Unit, for the investigation of the experimental error and for other advice, and to Dr. R. A. Kekwick, of the Lister Institute, for supplies of human thrombin and fibrinogen.

APPENDIX

Error of the Test

The thrombin clotting time was measured by one observer with various thrombin solutions in plasmas from 19 subjects (seven controls and 12 patients) over the range 8–30 sec.; 41 observations were obtained, each based on three to six replicate readings. The data were analysed to investigate the scatter among the replicates, as a measure of the experimental error of the test. It was found that the later replicates in a series tended to give a longer clotting time than the earlier, which was thought to reflect a deterioration in reagents. When this effect had been allowed for, the residual scatter was proportional to the mean clotting time, and the average coefficient of variation (the standard deviation expressed as a percentage of the mean) was about 3%. (This value agrees well with that of 5% previously published (Ingram and Armitage, 1952), which was derived only from the range of replicate readings without elimination of the trend and should thus be larger than the present estimate.) The coefficient of variation appeared to vary more among the patients than among the control subjects.

Despite the tendency for the clotting time to lengthen during the period of testing, an unbiased comparison beween two plasmas A and B can be obtained if the readings are made in such an order that the temporal effect is eliminated, as in Table IX (Cox, 1951). With this precaution, it was possible to devise a significance test for the difference between the mean readings from any two plasmas, using the estimate of sampling error from the analysis reported above. The procedure was to calculate the mean of all the readings from the two plasmas, the mean readings from each plasma, and the difference between the plasma means; the standard error of this difference was then a given percentage

TABLE IX

SEQUENCE OF REPLICATE READINGS FROM TWO SAMPLES

No. of Replicates	Order of Testing Plasmas A and B
2	AB, BA
4	AB, BA, BA, AB
6	AB, AB, BB, AA, AB, AB
8	AB, BA, BA, AB, BA, AB, AB, BA

These sequences are designed to eliminate systematic trends related to order of testing (such as progressive changes in reagents or observer facilitation or fatigue). The first sequence eliminates a linear trend (a steady drift throughout the period of observation); the second and third sequences eliminate also a quadratic trend (a steadily increasing drift); and the fourth sequence eliminates also a cubic trend (a more rapidly increasing drift). Clearly, balanced sequences cannot be obtained with odd numbers of replicates.

These sequences are generally applicable to all tests in which the subjects or treatments are compared by means of consecutive replicate readings and in which systematic trends might intrude.

(Table X) of the mean of all the readings. The significance of the difference between the means was tested by regarding the ratio of the difference to its standard error as a normal deviate: if the ratio exceeded 1.96, the probability of a greater difference arising by chance was less than 1 in 20 ($P<0.05$); if the ratio exceeded 2.58, the probability was less than 1 in 100 ($P<0.01$).

A significant difference does not necessarily imply a pathological difference, for significant differences are to be expected within the normal range. The value of the significance test is that it shows when apparent differences may be discounted on the grounds of experimental error alone.

Coefficients of variation which have been obtained from replicate readings of one-stage prothrombin times made by the same observer (for the experiment shown in Table V, for instance, the value was 1.78%) and by different observers have agreed well with the values obtained from this analysis for the thrombin clotting time. Thus the values given in Table X probably indicate the order of magnitude that might be obtained by other workers both for the thrombin clotting time and for the one-stage prothrombin time.

Further details of the investigation into the variability of the test results will be published elsewhere.

TABLE X

STANDARD ERROR OF THE DIFFERENCE BETWEEN TWO MEANS EXPRESSED AS A PERCENTAGE OF THE MEAN OF ALL THE READINGS

No. of Readings Contributing to Each Mean	Percentage Factor for Determining Standard Error of Difference between the Means
2	3·0
4	2·1
6	1·7
8	1·5

Example:
Patient's readings: 10.4, 11.2, 10.7, 11.0, mean 10.83 sec.
Control readings: 12.1, 11.6, 11.8, 12.2, mean 11.93 sec.
Grand mean: 11.38 sec. Difference between patient and control, 1.10 sec.

S.E. difference $= 11.38 \times \frac{2.1}{100} = 0.24$; $\frac{1.10}{0.24} = 4.6$. The difference is significant ($P<0.01$).

A more accurate estimate, and also values for unequal number of replicates and other instances not covered by this table, may be obtained from the formula:

$$\text{S.E.} = (0.03)\bar{y}\sqrt{\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}$$

Where n_1 and n_2 are the numbers of replicates making up the two series of readings (ordinarily, of course, $n_1=n_2$), $\bar{y}$ is the grand mean and S.E. is the required standard error of the difference between the means of the two series.

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DÖHLE BODIES IN THE LEUCOCYTES OF PATIENTS WITH BURNS

BY

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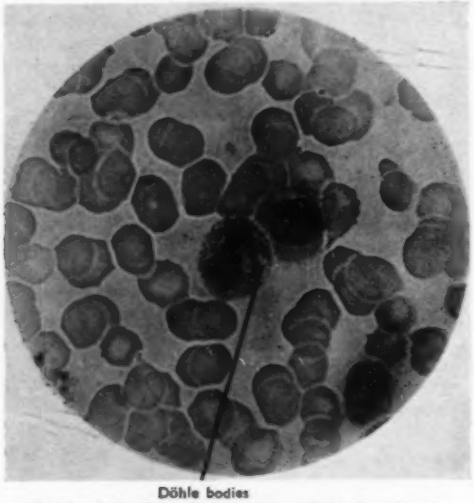
When working in a hospital for infectious diseases, Döhle (1911, 1912a and b) noticed round, blue-staining bodies in the cytoplasm of neutrophil leucocytes of patients suffering from scarlet fever. As these bodies were occasionally reminiscent of spirochaetes, Döhle first assumed them to be the cause of scarlet fever. This is not surprising if one remembers that only a short time before a spirochaete had been discovered as the cause of syphilis. However, the same bodies, which came to be known as Döhle bodies, were observed also in erysipelas (Kolmer, 1912; MacEwen, 1913–14), in diphtheria on about the third day (Kolmer, 1912), in typhus (MacEwen, 1913–14; Rehder, 1942), and possibly in tuberculosis (Bachman and Lucke, 1918). In measles Kolmer (1912) found them absent, while MacEwen (1913–14) considered their presence in 12 out of 35 cases as possibly associated with oral sepsis. Döhle bodies were described by Kolmer (1912) in a case of scarlet fever complicating burns.



Döhle bodies

FIG. 1.—A blood film from a patient suffering from burns.

The more modern staining techniques have so far added little to what Döhle clearly described. The bodies (Fig. 1) are usually round or oval shaped and have a diameter from just visible size to approximately 1 to 2 μ. They are found most often at the periphery of the cell. With Leishman-Giemsa, or any other of the usual Romanowsky stains, they are a sky blue to grey blue colour and are easily distinguished from the pinkish stained cytoplasm of the neutrophil leucocytes. Some of the cells contain one Döhle body, others may contain up to three or even four. They are always discrete and clearly separated by normal stained cytoplasm. Their staining properties, shape, and position in the cell distinguish them clearly from normal or pathological granulation occurring in neutrophil leucocytes.

The discovery of Döhle bodies in the neutrophil leucocytes of patients with burns was due to a chance observation when a blood film from a patient was examined for a quite different reason. In view of this finding, repeated blood films were taken from patients with burns of different severity in order to ascertain the frequency and timing of their occurrence and any possible association between the presence of Döhle bodies and other features such as the size of the burn, bacterial colonization, anaemia, or leucocytosis.

Material and Methods

Blood films were taken from 19 consecutive patients admitted to the Burns Unit of the Birmingham Accident Hospital between June 29 and August 15, 1953, and from nine patients with burns involving more than 13% of the body surface admitted later. Numerous cases have been investigated since then, but are not included in Tables I and II.

Blood films were prepared from capillary blood in the usual way about three times a week for the first three weeks and then weekly. They were stained by

Leishman-Giemsa. To avoid bias one of us was responsible for the collection of the clinical data and for the making of the films. The films were coded with serial numbers and examined by the other without knowledge of the origin of the film. At the end of the investigations the results were compared. Differential counts of at least 300 white cells were performed on a number of films. All other films were carefully examined for the presence of Döhle bodies. Structures found in the cytoplasm of the cells were counted as Döhle bodies, only if they conformed fully with all the characteristics given in the introduction. No note was made of their single or multiple occurrence in one or the same cell.

Results

Of the 19 consecutive patients admitted between the dates given above, 11 showed Döhle bodies at some time after their admission. The clinical details of these cases are shown in Table I. Of the 14 burns patients with 2% or less full-thickness skin loss, only six showed Döhle bodies: all five patients with more than 2% full-thickness skin loss showed Döhle bodies. Table I also includes nine patients admitted later, all with more than 13% full-thickness skin loss. All of these showed Döhle bodies. In two patients admitted to this hospital in 1955 the burns involving 10 to 14% full-thickness skin loss were primarily excised on the day of admission, and the grafts took almost perfectly. No Döhle bodies appeared in the leucocytes of these patients.

Inspection of Table I also suggests that there is a relationship between the incidence of Döhle bodies, pyrexia, bacterial colonization, and mortality. A direct association between the area of the burn and such clinical and pathological findings is already well recognized. This means that in order to seek for a positive association between Döhle bodies and a certain pathological finding, e.g., bacterial colonization, it is necessary to allow for the severity of the burn. The present evidence shows that Döhle bodies are more common in more extensive burns.

Döhle bodies have been found in patients whose burns were not colonized by group A streptococci, coagulase-positive staphylococci, coliform bacilli, or *Ps. pyocyanea*, and in patients who had not received any blood transfusions, plasma, or dextran. None of these factors alone would therefore appear to be essential by itself for the appearance of Döhle bodies.

Table II and Fig. 2 show the incidence of Döhle bodies at different times after burning. They have rarely been seen on the day of the burn, and the many abnormalities observed during the first hour after a severe burn, though on occasion somewhat

TABLE I

INCIDENCE OF DÖHLE BODIES IN 28 BURNS

	Patient	Total Blood Films during First Two Weeks	Films with Döhle Bodies	Area of Burn*		Total	Maximum Pyrexia (°F.) First 2 Weeks	Died	Bacterial Colonization of Burn First 2 Weeks in Hospital			
				Full-thickness Skin Loss	Partial Skin Loss				Group A H. S.	Coagulase +ve Staphylococci	*Ps. Pyocyanea*	Coliform Bacilli
19 consecutive cases	A	2	0	0	5	5	104	0	+	+	0	0
	B	2	0	0	5	5	99	0	0	0	0	0
	C	6	1	0	17	17	102	0	0	+	+	+
	D	6	0	<¼	0	<¼	99	0	0	0	0	0
	E	5	0	¼	0	¼	99	0	0	+	0	0
	F	2	0	0·3	0	0·3	100	0	0	0	+	+
	G	1	0	¾	6¼	7	102	0	+	0	+	+
	H	4	4	½	0	½	100	0	0	+	0	+
	I	4	3	½	9½	10	101	0	0	+	+	+
	J	3	1	<1	13+	14	100	0	0	+	0	+
	K	6	0	1	4	5	102	0	0	+	0	0
	L	5	3	1	2	3	103	0	+	+	+	+
	M	5	4	1	14	15	103	0	0	+	+	+
	N	1	0	2	4½	6½	104	0	+	+	0	+
	O	4	3	4	6	10	100	0	0	+	+	+
	P	5	3	5	15	20	100	0	0	+	+	+
	Q	6	2	7	13	20	103	0	0	+	+	+
	R	6	4	12	13	25	103	0	0	+	+	+
	S	5	5	14	0	14	103	+	0	0	+	+
9 further cases selected because of large area of burn	T	5	4	14	21	35	104	0	0	+	+	+
	U	8	7	20	5	25	104	0	0	+	+	+
	V	6	5	20	5	25	106	0	+	+	+	+
	W	3	2	25	2	27	104	0	0	+	0	+
	X	2	2	27	16	43	103	0	0	+	+	+
	Y	5	4	40	10	50	102	0	+	+	0	+
	Z	3	3	Not known		60	103	+	0	+	+	+
	AZ	3	2	Not known		70	104	+	0	+	0	+
	BZ	4	4	70	5	75	101	+	0	+	+	+

* Each group ranked in order of area of full-thickness skin loss

TABLE II
INCIDENCE OF DÖHLE BODIES AT DIFFERENT PERIODS AFTER THE BURN

Patients (see Table I)	Blood Films with Döhle Bodies	Days							Weeks			
		1	2	3	4	5	6	7	2	3 & 4	5 & 6	>6
19 consecutive cases	+	0	2	1	5	2	2	2	14	4	0	0
	0	1	9	1	5	2	7	0	23	20	9	8
	Total	1	11	2	10	4	9	2	37	24	9	8
9 further cases selected because of large area of burn	+	2	2	4	6	4	2	3	9	9	3	2
	0	4	1	0	0	0	0	0	1	2	2	11
	Total	6	3	4	6	4	2	3	10	11	5	13
Total 28 cases	Proportion positive	2/7	4/14	5/6	11/16	6/8	4/11	5/5	23/47	13/35	3/14	2/21

obscuring the picture, were certainly not obscuring the presence of Döhle bodies. Somewhat more frequently than on the day of the burn, they were seen 24 hours afterwards and usually reached their highest incidence by the third day.

Repeated blood films have been taken during the first 12 hours in a very extensive burn (80% of the body surface). Döhle bodies were not definitely seen until the twelfth hour, although there was a primary leucocytosis of 47,000 white cells per cubic millimetre by the sixth hour.

In many cases Döhle bodies have disappeared by the third week, but in more extensive burns they may persist for six weeks or more (Fig. 2, Table II). Döhle bodies frequently disappear at about the time of near complete skin cover and clinical convalescence of the patient.

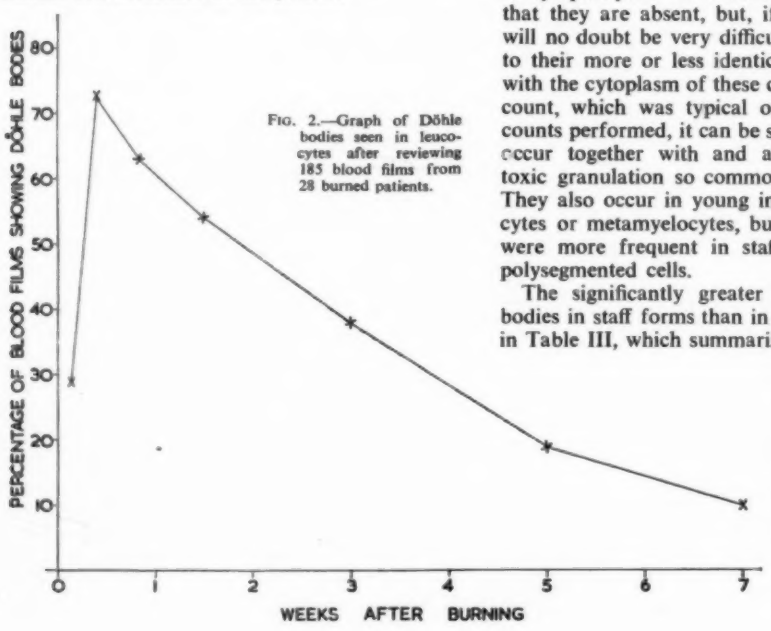


FIG. 2.—Graph of Döhle bodies seen in leucocytes after reviewing 185 blood films from 28 burned patients.

A typical blood count in a fairly severely burned patient (25% of body surface) gave the following values on the third day after injury:

Myelocyte containing Döhle bodies but no toxic granulation	0·3%
Metamyelocyte with no toxic granulation or Döhle bodies	5·3%
" " toxic granulation but no Döhle bodies	1·3%
" " Döhle bodies but no toxic granulation	2·7%
" " toxic granulation and Döhle bodies ..	0·3%
Polymorphs with no toxic granulation or Döhle bodies ..	11·7%
" " toxic granulation but no Döhle bodies ..	2·7%
" " Döhle bodies but no toxic granulation ..	7·0%
" " toxic granulation and Döhle bodies ..	3·3%
Staff forms with no toxic granulation or Döhle bodies ..	11·3%
" " " toxic granulation but no Döhle bodies ..	4·7%
" " " Döhle bodies but no toxic granulation ..	7·3%
" " " toxic granulation and Döhle bodies ..	2·3%
Lymphocytes	15·0%
Eosinophils	Nil
Basophils	Nil
Monocytes	23·3%
Plasma cells	1·3%

Döhle bodies were not recognized in monocytes or lymphocytes. This does not necessarily mean that they are absent, but, if they do occur, they will no doubt be very difficult to recognize owing to their more or less identical staining properties with the cytoplasm of these cells. From the above count, which was typical of a number of other counts performed, it can be seen that Döhle bodies occur together with and also independently of toxic granulation so common in burned patients. They also occur in young immature cells (myelocytes or metamyelocytes, but see also below) and were more frequent in staff forms than in the polysegmented cells.

The significantly greater frequency of Döhle bodies in staff forms than in polymorphs is shown in Table III, which summarizes counts of 15 films from patients with burns.

We did not feel justified in performing marrow punctures on these patients, as some of them were too ill and most of them were children. We had,

TABLE III

DIFFERENTIAL COUNTS (300 CELLS) OF 15 FILMS FROM SEVEN PATIENTS WITH BURNS INVOLVING 5–25% OF THE BODY AREA SHOWING INCIDENCE OF DÖHLE BODIES IN STAFF FORMS AND POLYMORPHONUCLEAR LEUCOCYTES

Patient	Days after Burn	Polymorpho-nuclear Leucocytes	Polymorphs with Döhle Bodies	% of Döhle Bodies in Polymorphs	All "Staff" Forms	"Staff" Forms with Döhle Bodies	% of Döhle Bodies in "Staff" Forms	Higher Incidence of Döhle Bodies in
A	7	25·0	10·0	40·0	9·3	5·0	53·8	Staff forms
O	1	75·9	0·3	0·4	7·6	0·3	4·0	" "
O	3	64·3	1·3	2·0	5·9	0·3	5·1	" "
P	3	67·4	11·0	16·4	16·9	5·6	33·1	" "
P	6	55·9	16·6	29·7	24·7	11·0	44·5	" "
P	8	51·6	4·3	8·3	20·3	3·0	14·8	" "
Q	10	44·1	2·7	6·1	14·1	0·7	5·0	Polymorphs
Q	12	25·0	13·0	52·0	28·1	18·9	67·3	Staff forms
R	3	61·0	9·0	14·8	18·0	7·3	40·6	" "
R	5	60·0	30·7	51·1	15·8	8·4	53·1	" "
R	7	49·3	15·3	31·0	32·9	14·3	43·5	" "
S	1	78·7	0·7	0·9	9·6	0·3	3·1	" "
S	3	24·7	10·3	41·7	33·5	12·9	38·5	Polymorphs
S	5	28·4	23·7	83·4	34·7	33·1	95·4	Staff forms
Not in Table I	35	22·6	1·0	4·4	2·0	0·3	15·0	" "

The χ^2 distribution of Döhle bodies between staff and polymorphonuclear leucocytes shows a highly significant preponderance in the staff forms ($P = < 0{\cdot}001$)

however, occasion to examine marrows from two patients shortly after their death. On examination of the films obtained, one gained the impression that the myeloid series showed a fairly gross disturbance of maturation. Otherwise typical myelocytes showed a blue cytoplasm in spite of the fact that their granulation and nuclei were already fully mature. Some metamyelocytes showed big patches of blue cytoplasm on many occasions. It is conceivable that Döhle bodies as found in the more mature cells are the remains of these patches of blue cytoplasm. However, the films had been taken after death and, as it is well known that very rapid changes occur in the marrow cells after the death of the patient, it would not appear to be safe to base any conclusions on these observations.

Discussion

The finding of Döhle bodies in burned patients was unexpected. They had been seen previously, as already pointed out, in scarlet fever, other streptococcal infections, diphtheria, typhus, and tuberculosis. Kolmer (1912) has described the occurrence of Döhle bodies in scarlet fever which complicated burns, but he regards their occurrence as due to the scarlet fever and does not discuss any connexion with the burn. Morphologically, the bodies seen in the films taken from burned patients were identical with those obtained in scarlet fever. They were more common in patients with extensive full-thickness skin loss. They appeared during the first few days and persisted for two or more weeks after burning, often until near complete skin cover had been obtained. This timing is very similar to the acute anaemia of burns which is due largely to an unexplained disappearance of red cells ; but scanty numbers of Döhle bodies have been seen in patients with burns of too small an area for the haemoglobin to fall below 11.8 g. %. However, still more data are required to establish the significance of the association between Döhle bodies and area of remaining full-thickness skin loss or degree of anaemia.

The appearance of Döhle bodies on the second or third day does not coincide with the height of the total neutrophil leucocyte count in the peripheral blood, but it is possible that it does coincide with the first rapid new formation of leucocytes. Leucocytes disappear into the burned area within the first few hours, but dense polymorphonuclear leucocyte infiltration may take one or two days to form in the burn area (Gordon, Hall, Heggie, and Horne, 1946 ; Moritz, 1947), and massive purulent exudate (present even in burns from which no bacteria were cultivated) is usually not seen on the burned surface until the third day. It is possible, therefore, that the fall in the total polymorphonuclear leucocyte count often seen in the peripheral count on the second or third day (Van Duyn, 1945 ; Sevitt, 1951) is associated with an increased rate of formation of leucocytes and an even more increased rate of loss of leucocytes into the burned area. This is the time of the appearance of Döhle bodies in the leucocytes of the peripheral blood.

A common cause of the occurrence of these marked changes in the cytoplasm of leucocytes has not been found in the diseases in which Döhle bodies have already been reported, although most were associated with a polymorphonuclear leucocytosis. Any "new" morphological change noticed always poses the questions, What is it, what is the cause underlying it, and what is its clinical and pathological significance ? None of

these questions can be answered with the present evidence, but it is hoped that our observation may lead to further investigation into the problem.

It may be that the presence of Döhle bodies reflects one of the many changes in biochemistry of the neutrophil leucocytes taking part in an acute reticulo-endothelial response. In all cases reported so far (with the possible exception of typhus fever) the appearance of Döhle bodies coincided more or less with the appearance of a polymorphonuclear leucocytosis. Histochemical and biochemical investigations on these leucocytes may possibly give results which would clarify the problem of their causation. From the morphological point of view, a very precise knowledge of their incidence in different diseases might increase our knowledge of their pathological significance. For example, it is not known whether they always occur in bacterial infections which produce a leucocytosis or whether they may on occasion also appear in leucopenic pictures. It is not known how often there is an associated unexplained disappearance of red cells. On occasion it has been claimed that leukaemic leucocytes have never shown Döhle bodies, whereas other observers have described Döhle bodies occurring in myelogenous leukaemia. They have, to our knowledge, not been described in pernicious anaemia or haemolytic anaemias and nothing is known of their appearance in the reticuloses. As Döhle bodies are easily overlooked, it may perhaps be rewarding to look out for them regularly in conditions where their occurrence has not been described or suspected.

Summary

Döhle bodies have been found to be frequently present in the neutrophil leucocytes of patients with burns involving more than 2% of full-thickness skin loss. They appear during the first day or two after the burn and often disappear when complete or nearly complete skin cover is obtained. Their clinical and pathological significance is as yet unknown. It is suggested that a more precise knowledge of their incidence in different diseases might add to our knowledge of their pathological significance.

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NOTES ON A SERUM CONTAINING ANTI-P IN HIGH TITRE

BY

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In 1927 Landsteiner and Levine reported the discovery in human blood of the antigen P. Since this time, the corresponding antibody has been detected frequently, but, in most of the sera described, is weak both in titre and avidity, and requires a special technique in order to demonstrate its presence. For this reason the unexpected finding of a powerful naturally occurring antibody, anti-P, in the serum of a patient is of interest. The following is a description of the reactions of the antibody which we found during routine work.

Observations on the Case

On March 4, 1954, S. W. W., a boy aged 12 years, was listed for operation because of a hydatid cyst of the lung. He was found to be blood group A Rh positive, and his serum was then cross-typed with the pilot cells of four bottles of group A Rh-positive blood in the usual way in order to have blood available for the operation. It was found that the cells of all four bottles of blood were agglutinated, the agglutination becoming more obvious after the cell suspension had been taken out of the incubator for examination.

A fresh specimen of blood (20 ml.) was obtained from the patient immediately in order to investigate the reactions of the serum. The blood group was checked and found to be group A (subgroup A_1) Rh positive. On checking the blood groups of the four bottles of blood, three were found to be A_1 Rh positive and one A_2 Rh positive. The patient's serum was then found to agglutinate the cells of 51 out of 68 bottles of blood of groups A Rh positive, A Rh negative, O Rh positive, and O Rh negative against which it was tested, but sufficient compatible group A Rh-positive blood was obtained for the operation, which took place at the appointed time.

It was noted that the agglutination became more obvious at 8° C. than at 37° C. Nevertheless, the undiluted serum gave a very strong agglutination in the incubator as well as at room temperature. The titre of the antibody, using cells suspended in serum albumin solution and in Rous Turner solution for a period of one hour, was as follows :

Temperature of Test (° C.)	Cells in Rous Turner Solution	Cells in Albumin
37	4	32
8	64	256

At room temperature the diluted serum caused agglutination of cells suspended in Rous Turner solution in the following times.

Titre	Time
1	30 sec.
2	60 ,,
4	2 min.
8	5 ,,
16	8 ,,
32	15 ,,
64	75 ,,

Gene Frequency of Antigen.—The serum of S. W. W. was tested against 92 samples of group A Rh-positive blood chosen at random. Of these, 22 were found to be compatible and 70 were not compatible. The gene frequency corresponding to the antigen which was reacting with the antibody in S. W. W.'s serum and the gene frequency of its presumed allelomorph was worked out using the method described in M.R.C. Memorandum No. 27 (Mollison, Mourant, and Race, 1952). These worked out as 0.51 and 0.49 respectively. The panel of blood used is too small for accurate determination of the gene frequencies, but, as the gene frequencies of P and p are 0.4901 and 0.5099 respectively, the results suggested that we might be dealing with the P blood group and that the antibody was anti-P.

Tests Against Panel of Known Blood Groups.—The serum of S. W. W. was tested against the red cells of 19 individuals in whom the ABO, MNS, P, Lewis, Rh, Duffy, and Kell blood groups were known. Fourteen were P positive, and the cells of these agglutinated with the serum of S. W. W. Four of the panel were P negative and the serum did not agglutinate the cells of these individuals. The red cells of

one of the donors on the panel, A. D., who had been notified as "P negative," were agglutinated by the serum of S. W. W. Accordingly, the blood of A. D. was sent to the Commonwealth Serum Laboratories, Melbourne, so that the cells could be retyped by Mr. R. T. Simmons, who confirmed that A. D. did in fact belong to blood group P : the initial error in grouping the cells was most probably due to low potency anti-P sera. The serum of S. W. W. was then retested against known P-positive and P-negative blood in the Commonwealth Serum Laboratories. The serum agglutinated all "P-positive" cells and failed to agglutinate all "P-negative" cells.

Family Studies.—The family of S. W. W. were interested in the peculiarity of S. W. W.'s blood and were cooperative during the investigations. There are eight members in the family, including S. W. W., his father, mother, two brothers, and three sisters. S. W. W., born in 1941, was the fourth child in the family. He had been immunized against diphtheria in 1942. In 1949 he had penicillin and streptomycin injections for complications of his lung condition, and in 1952 had both active and passive anti-tetanus immunization. He had never had a blood transfusion or injection.

Only the mother of S. W. W. was found to be P positive (by testing the cells with the serum of S. W. W.). All the other members of the family are P negative (including S. W. W. himself) ; their cells do not agglutinate with the serum of S. W. W.

The serum of each member of the family was tested against known P-positive and P-negative cells. In no case did agglutination occur, indicating that no member of the family other than S. W. W. had produced the antibody, anti-P, even though they were all (with the exception of S. W. W.'s mother) P negative.

Source of the Antibody.—As S. W. W. had not been transfused previously, it was thought that he might have been immunized by the inoculations he had received. Accordingly, serial dilutions of the serum of S. W. W. were made from 1 to 64, and to each serial dilution was added an equal quantity of diphtheria prophylactic, formalized tetanus toxoid, and tetanus antitoxin. After allowing one hour to elapse during which time any reaction could be expected to have taken place, a drop of P-positive cell suspension was dropped into the mixture and the tube noted in which agglutination took place. It was found that in each case agglutination took place in the same tube as the saline control, suggesting that there was no P antigen in the three materials tested and that S. W. W. had not been stimulated to produce anti-P antibody as a result of previous inoculation.

Absorption and Elution.—Using the method of Mollison (1951), the serum of S. W. W. was absorbed with the cells of one of the hospital staff, A. W., who was group O Rh positive, P positive. The absorbed serum was found to have a much weaker agglutinating titre than unabsorbed serum.

The cells of A. W. with the absorbed antibodies were then shaken in saline and the antibody was eluted in the manner described by Mollison. The eluate agglutinated two specimens of P-positive cells, whereas the absorbed serum failed to agglutinate the same cells. It is possible, therefore, to obtain this antibody in a pure form.

Discussion

Approximately 75% of white people are P positive, belonging to the genotype PP or Pp. The remainder are P negative and belong to the genotype pp. The gene p is allelomorphic to P. Due to the fact that transfusion is so widely practised, and as anti-P serum is not available in adequate quantity for the routine grouping of donors and recipients, many P-negative people are transfused with P-positive blood. As a result these recipients could develop anti-P. Transfusion reactions from this cause have indeed been reported. However, most anti-P sera are of low titre and the reactions are generally mild.

The present case indicates that the antibody anti-P may on rare occasions be present in sufficient titre to be a cause of a severe transfusion reaction. Recently Kölbl and Speiser (1953) have reported a case in which the maternal anti-P antibody had a titre of 1 in 16 in human serum at body temperature. The case history of her infant suggests that it suffered from erythroblastosis foetalis due to the presence of anti-P in the maternal blood as a result of maternal immunization.

The need for careful routine cross-typing of donor cells against patient's serum is borne out by this case. Atypical isohaemoglutins occur rarely, but they make their appearance with dramatic suddenness at inconvenient times and it is therefore essential that a rigid technique should always be followed of grouping and cross-typing each patient's serum against each blood bottle issued to him before notifying it as compatible.

We would like to thank Mr. R. T. Simmons, Commonwealth Serum Laboratories, Melbourne, for his assistance in performing some of the serological tests in this case, and the parents and family of the patient for their willing cooperation during the investigation.

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A STUDY OF NAPHTHIONIN, A NEW HAEMOSTATIC DRUG

BY

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The emphasis has shifted in recent years from arbitrary methods used in the treatment of bleeding diseases to the search for the individual factors underlying these conditions. The haemorrhagic diathesis in haemophilia, hypoprothrombinaemia, Christmas disease, and others can now be treated with the specific clotting factor found to be deficient.

There remain a number of conditions, e.g., peptic ulceration, operative surgery, obstetrical haemorrhages, in which there is danger to life from bleeding although the coagulation process is not at fault. The acceleration or promotion of haemostasis in these states would be of the greatest benefit.

Esteve, Oriol, and Regné (1949) employed "naphthionin" (sodium-alpha-naphthylamine-4-sulphonate) in an effort to produce a substance which would be of use in the prophylaxis and treatment of vascular haemorrhage. Naphthionin is related to Congo red, which had been claimed to be of value in the treatment of the haemorrhagic tendency in thrombocytopenic purpura (Brühl, 1933).

The effects of "naphthionin" *in vitro* and *in vivo* on a group of healthy adult males have been assessed in this paper.

Methods

Lee and White (1913) Clotting Method (Modified).—Blood (5 ml.) was withdrawn into a dry syringe from an antecubital vein of a healthy adult in the minimum time after the application of a tourniquet. The whole process was accomplished in under 30 seconds. Three 1 ml. amounts of blood were delivered into three 3 × ¾ in. test-tubes which had been allowed to warm to 37° C. in a water-bath.

The residual 2 ml. of blood in the syringe was discarded to reduce contamination by tissue juices rich in thromboplastin. As soon as the first blood had entered the syringe timing was begun with a stopwatch.

The first tube was tilted gently at 30-second intervals until a firm, totally adherent clot formed, not being dislodged by complete inversion of the tube. This was regarded as the end-point in the clotting reaction and the same procedure repeated on the second and third tubes.

Prothrombin Estimations.—Prothrombin times were based on the Quick single-stage principle (Quick, 1944) using a human brain thromboplastin extract. This gave a normal reading of 12 to 14 seconds using 1% potassium oxalate as the anticoagulant and 0.2% calcium chloride.

Bleeding Times.—The method of Duke (1915) was employed. Blotting was performed at 15-second intervals.

Heparin-retarded Plasma Clotting Times.—The method of Poller (1954) was used. A slight deviation from the technique previously described was the performance of the test 30 minutes after collection. This has resulted in longer clotting times owing to the reduction of the effects of storage of oxalated plasma in glass.

Inhibited clotting methods whether by the use of silicone glassware or heparin are attempts to achieve more sensitive tests for the clotting mechanism than those in routine clinical use.

Results

In Vitro Tests.—Naphthionin was diluted 60 times and 0.1 ml. containing 1/6,000 g. was added to the 3 × ¾ in. test-tubes employed for the Lee and White clotting test. This gave a level roughly equivalent to blood level after a 1 g. injection of blood had been delivered for the Lee and White test.

To a second tube 0.1 ml. of normal saline was added as a control before the addition of blood for the clotting test.

TABLE I

EFFECT OF NAPHTHIONIN AT EQUIVALENT BLOOD CONCENTRATIONS ON THE LEE AND WHITE CLOTTING TEST *IN VITRO*

Number of Subjects Tested	Average Clotting Time	
	Blood and Naphthionin	Blood and Saline
6 males	4·2 min.	4·0 min.

It appears that " naphthionin " has no dramatic immediate effect on whole blood clotting times.

Response to Intravenous Naphthionin in Healthy Adult Males.—The effect of the intravenous administration of 10 ml. (1 g.) " naphthionin " has been observed in 10 healthy young adult males.

Before injection the bleeding time, clotting time (by the modified Lee and White method), prothrombin time, and the heparin retarded clotting time were estimated.

Ten millilitres (1 g.) of the drug was injected. Bleeding times, clotting times, and prothrombin times were repeatedly tested at 30-minute intervals over a three-hour period while heparin-retarded tests were repeated hourly.

The effect on bleeding, clotting, and prothrombin times may be observed in Figs. 1 and 2.

The significant result was the appreciable reduction of the bleeding time, which appeared to be greatest one hour after injection. The bleeding time is a crude test, yet the differences noted are reasonably marked. The mean deviation is 0.775, which is significantly different from zero ($P<1\%$).

TABLE II

EFFECT OF INTRAVENOUS ADMINISTRATION OF NAPHTHIONIN ONE HOUR AFTER INJECTION OF 1 G. (10 ML.)

Total Subjects	Modified Lee and White Test		Bleeding Time		Prothrombin Time		Heparin Clotting	Plasma Time
10	Initial 7·3 min.	1 hr. later 7·36 min.	Initial 2·35 min.	1 hr. later 1·37 min.	Initial 14 sec.	1 hr. later 14 sec.	Initial 14·3 min.	1 hr. later 14·9 min.

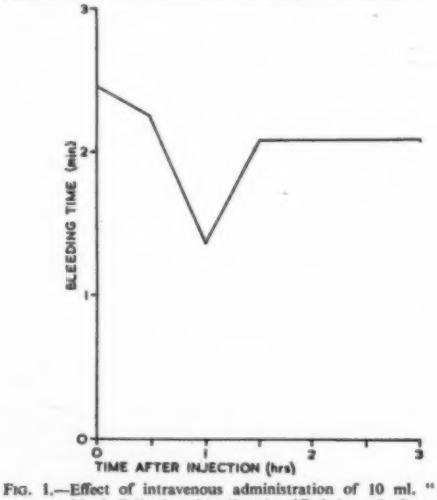

FIG. 1.—Effect of intravenous administration of 10 ml. " naphthionin " (1 g.) on bleeding time (Duke's method).

No significant change was observed in the clotting times—heparin-retarded plasma clotting or prothrombin times.

Discussion

Several papers have been published describing the clinical benefit of the drug in the prevention and treatment of haemorrhage (Dubois-Ferrière, 1950; Puigvert, Cols, Mas-Oliver, and Noguera, 1951; Greif, 1952; Rambauske, 1953; Pirquet, 1954). It has now been made generally available in this country.

Hitherto no adequate attempt to assess the action of the drug has been reported.

The present findings do not support the view (based mainly on capillary clotting times in rabbits) that " naphthionin " in a dosage of 1 g. (10 ml.) causes a marked reduction in blood coagulation times in man.

Naphthionin lowers the iso-electric point of plasma proteins, including fibrinogen, with the result that in blood with a *p*H in the region of 7.3 the passage from sol to gel state is facilitated.

The tendency to gel formation was observed in the course of performing the Lee and White tests, and, unless the formation of a firm, totally adherent clot had been regarded as the end-point, this would have caused difficulty with readings. The

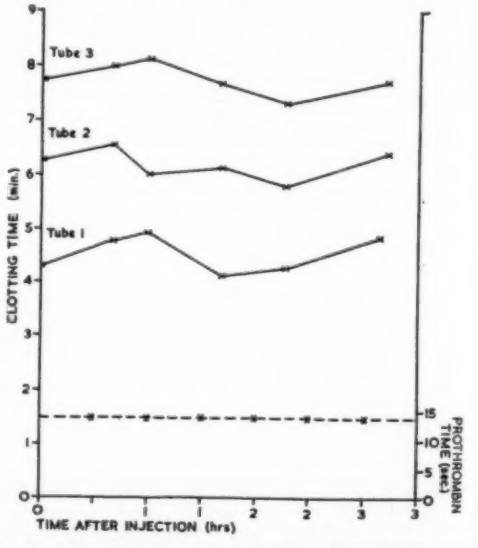

FIG. 2.—Average graph based on six normal adults, showing the effect of intravenous administration of " naphthionin " (10 ml. = 1 g.) on the modified Lee and White clotting time, and of prothrombin by the Quick single-stage method.

formation of a gel would have been particularly prone to give low readings in the outmoded capillary and slide clotting methods which previously have been used to investigate this drug, and is perhaps the reason why shortening of clotting times was observed with these unreliable techniques (Dubois-Ferrière, 1950).

Conclusion

Naphthionin has an appreciable effect on haemostasis as measured by bleeding times in man but no appreciable effect on blood clotting. The mechanism of this action may be due to the tendency to gel formation resulting from the lowering of the iso-electric point of fibrinogen with the result that small capillary punctures are more easily sealed off.

Summary

A study has been made of a new haemostatic drug, "naphthionin," which has recently been made generally available in this country.

This drug causes marked reduction of bleeding times but no effect on blood coagulation as measured by the modified Lee and White clotting time, the heparin-retarded plasma clotting time, or prothrombin time.

The haemostatic action of "naphthionin" appears to be an effect on the smaller vessels and is possibly due to the tendency to gel formation resulting from the lowering of the iso-electric point of fibrinogen nearer to blood *p*H level by "naphthionin."

Samples of "naphthionin" were generously supplied by Maw and Sons Ltd.

Dr. L. Stent, Withington Hospital, Manchester, provided the human brain thromboplastin employed in the prothrombin estimations.

I am indebted to Dr. Cullumbine for encouragement in this work.

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THE DEMONSTRATION OF THE IRON-BINDING GLOBULIN (TRANSFERRIN) IN SERUM AND URINE PROTEINS BY USE OF ^{59}Fe COMBINED WITH PAPER ELECTROPHORESIS

BY

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The observation by Cartwright, Gubler, and Wintrobe (1954) that patients with the nephrotic syndrome often have a markedly reduced serum iron level associated with a lowered iron-binding capacity and an increased urinary excretion of the metal is in harmony with the theory that in this condition there is a loss of the iron-binding protein, together with other serum proteins, through the kidneys. It is now well established that the vehicle for iron transport in the body is a protein of relatively low (90,000) molecular weight (Surgenor, Koechlin, and Strong, 1949). This protein was at one time (Cohn, 1947) thought to be less specific than has since proved to be the case. It was formerly thought probable that the transport of copper, and perhaps of zinc too, was also a function of this fraction because it can be demonstrated *in vitro* that the iron-free metal-combining globulin, as it was then termed, is able to form a complex with copper.

These two metal-protein complexes, however, have different colours, the iron pink and the copper yellow. Holmberg and Laurell (1947) have described how the yellow of the copper complex was changed immediately to the pink of the iron complex on adding an iron salt to its solution. The same authors have shown that practically all of the serum copper is accounted for as the copper-containing enzyme coeruloplasmin. Their observations, including a study of the behaviour of zinc ions in competition with iron and copper for the protein, have enabled them to conclude that the sole function *in vivo* of the "metal-combining" globulin is the transfer of iron. They have given it the name "transferrin," and the preparation of its complex with iron in a crystalline state has been recently described by Laurell (1953).

The low molecular weight of this protein causes it to resemble albumin in its solubility properties, although on electrophoresis it has the mobility of a β-globulin. It is not possible by paper electrophoresis at *p*H 8.6 in barbitone buffer to distinguish between this globulin and others in the β-fraction. Wallenius (1952) demonstrated that when the rat is the experimental animal the addition of radioactive iron *in vitro* to the animal's serum led to the appearance of radioactivity in the β-globulin region after electrophoresis on paper. A similar result was obtained if the iron were administered orally or parenterally and the blood withdrawn afterwards. Addition *in vitro* to human serum produced comparable results. The latter technique is employed in work reported here, the results of which have been referred to in part by Hardwicke (1954). By this simple means it has been shown that in two of the pathological conditions in which proteinuria occurs, viz., the nephrotic syndrome and congestive cardiac failure, transferrin is lost into the urine. During the course of these experiments it became clear that the reaction between iron and transferrin is very highly specific and this specificity is not impaired in the presence of a variety of serum protein abnormalities.

Since the completion of this work Horst (1954) has reported the finding of an iron binding β-globulin in ascitic fluid, pleural exudate, and in nephrotic urine.

Apparatus

The apparatus used in this study was of the hanging-strip pattern, after Durrum (1950). It was made in such a way as to avoid the disadvantage often met in this type, namely, the line of contact between filter paper and the horizontal rod or bar used to support it. By suspending the paper by the edges over glass rods bent to form semicircles it is possible completely to avoid contact of the protein bands with the support. The apparatus is illustrated in Fig. 1. Two strips of paper, Whatman No. 100 6.25 × 45 cm.,

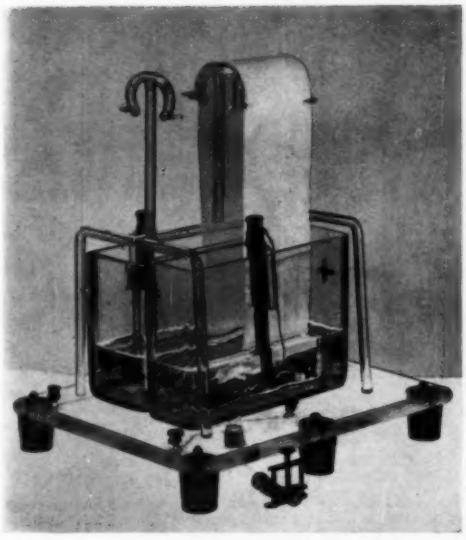

FIG. 1.—Illustration of paper electrophoresis apparatus.

can be accommodated side by side and direct current is supplied from a power pack giving a maximum of 450 volts. A large rectangular glass jar (not shown) covers the apparatus when in use. An inert gas may be admitted through the base when required.

Materials and Methods

The tracer iron used, ^{59}Fe, was in each case of a high specific activity. An early batch had an activity of 0.4 $\mu C/\mu g.$, which had been improved in batches some two years later to 3.8 $\mu C/\mu g.$ It was obtained as a solution of the chloride *ca.* *p*H 2 from the Atomic Energy Research Establishment at Harwell.

Urine and blood were collected from patients with the nephrotic syndrome and with proteinuria arising from congestive cardiac failure. To the separated serum was added ^{59}Fe to a concentration of either 0.5 or 0.05 $\mu g./ml.$ and a little solid ascorbic acid (about 5 mg./ml.). Similar additions were made to the urine, which had been first concentrated by dialysis in a collodion sac against hypertonic "dextran" solution of M.W. about 100,000. The amount of iron added was the minimum possible to obtain a satisfactory autoradiograph. By taking a larger volume of the unconcentrated urine it was found that, under the conditions used, no appreciable amount of protein was lost by diffusion through the membrane during dialysis. For the production of a urine protein separation more comparable with serum, however, it was found preferable to concentrate it first.

Of the serum-iron and urine-iron mixtures the amounts used for electrophoresis were the equivalent of 0.05 ml. of serum or concentrated urine respectively. These small volumes (0.055–0.06 ml.) were spread by means of a micropipette along a pencil line drawn across the middle of a strip of filter paper, and to within approximately 1 cm. of the edges. The strip was then placed in the glass frame and buffer solution (barbitone *p*H 8.6, 0.05M Na barbitone, 0.01M barbitone) applied to the paper on each side just below the protein solution. It was allowed to ascend the last centimetre by capillarity. A current of 1.5 mA per strip was allowed to pass for 16 to 18 hours.

After electrophoresis the strips were dried in an oven at 100 to 105° C. and then placed in contact with Kodak "industrex D" *x*-ray film. Length of exposure varied from two to three weeks, after which the proteins were stained with bromphenol blue.

Results

During earlier experiments (Neale and Besford, unpublished) it had been found that both ferric and ferrous iron would enter the same position, but that uptake into the β-globulin was quicker and more complete if the iron were reduced. This is in agreement with Laurell's conclusions (1947) which were drawn from observations based on a purely chemical technique. Accordingly, a small amount of ascorbic acid, approximately 2 mg./0.5 ml., was added to the serum and urine to ensure optimal acceptance of the iron. The quantity of iron added to the serum was designed to be within the binding capacity, and a similar amount was added to the urine.

The results of electrophoresis and autoradiography are shown in Figs. 2 and 3. In each case the autoradiograph is placed to the right of its corresponding strip. Sharp black bands, the result of radioactivity, occur opposite the β-globulin bands. In Fig. 2 (b) (serum) this radioactive band is considerably sharper and more easily distinguished than the β-globulins themselves. In all four sera the added iron has been completely taken up, but three of the four urines exhibit areas behind the β-globulins which show evidence of radioactivity. This is a typical "overload" picture. Iron has been added in excess of the carrying power of the transferrin and this excess has behaved as would a solution of ferrous chloride in an oxidizing atmosphere in the absence of any protein. Under these conditions iron travels towards the anode, not the cathode (cf. Wallenius), presumably because in the slightly alkaline condition existing the hydroxide produced in the form of a sol adsorbs hydroxyl ions and becomes itself negatively charged. This charged sol is in part precipitated on the paper and in part moves in the same direc-

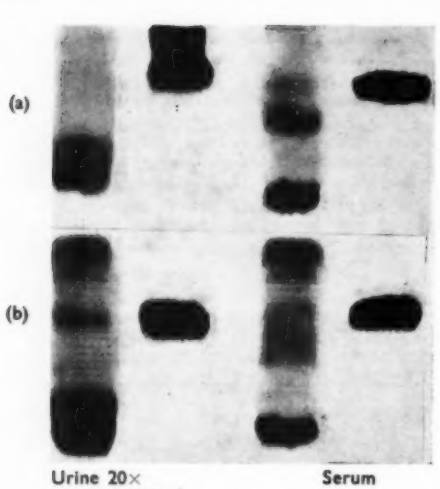

FIG. 2.—Addition of ^{59}Fe to serum and urine in two cases of the nephrotic syndrome, in these instances of 0.5 μg. (activity 0.2 μC) to each millilitre of serum or concentrated urine.

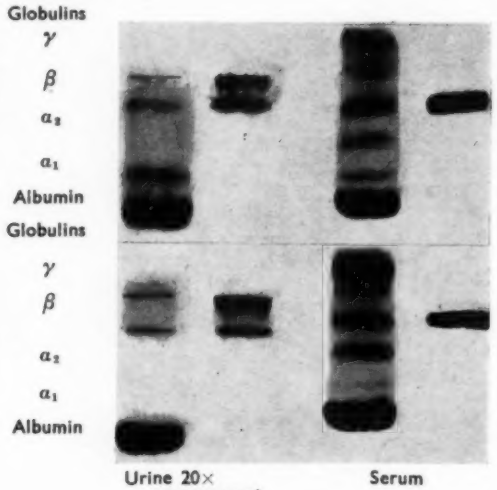

FIG. 3.—Addition of ^{59}Fe to serum and urine in two cases of congestive cardiac failure, in these instances of 0.05 μg. (activity 0.2 μC) to each millilitre of serum or concentrated urine.

tion as the protein until oxidation occurs. A solution of ferric chloride under similar conditions is found to mordant firmly on to the paper where it is applied, although it seems to be protected somewhat by the presence of protein and can then also leave a trail. The result observed, therefore, is a combination of the tail of activity produced as described, and the sharp band corresponding in position to the transferrin.

This overloading could have occurred because of the relatively smaller amount of transferrin in the urine concentrates, and the only urine showing complete uptake (Fig. 2b) has a β-globulin band comparable in density to its corresponding serum when stained with bromphenol blue.

Although care was taken to avoid contamination of the urine, any traces of iron introduced during the collection and preparation of the specimens would be "scavenged" and held by the transferrin, partly blocking the entry of the ^{59}Fe.

Under the conditions described there is no interchange of added iron with the iron already present in complex form and where radioactivity is taken up it is indicative of the presence of unsaturated transferrin. This has been verified by adding 0.05 μg. ^{59}Fe to 1 ml. of serum withdrawn from a patient suffering from haemachromatosis whose serum iron concentration was 303 μg. per 100 ml. In this case there was only a very small proportion of the activity associated with the β-globulin band in spite of the extremely small quantity of iron added, and the autoradiograph showed almost the same picture as the control without serum.

The specificity of transferrin can be demonstrated with any serum specimen by deliberately adding iron in excess of the saturation limit, when it is seen that only transferrin accepts it, and that the excess is not, under these experimental conditions, attached to any other protein. An addition of about 2 μg. Fe per ml. is accepted by many sera before overloading, which agrees with the unsaturated binding capacity found by Laurell and other workers.

It is found that the presence of the abnormal protein found in the serum in multiple myelomatosis has no interfering effect on the iron-binding function of transferrin *in vitro*. This is true also of the large increase in α_2-globulin which occurs in the nephrotic syndrome, and a patient with very severe hypogammaglobinaemia was found to exhibit normal iron binding. The specificity of the reaction between transferrin and iron in the presence of these several abnormal conditions argues that the appearance of an iron-linking protein having the mobility of a β-globulin in the urine of patients with proteinuria represents the leakage of transferrin through the kidneys. This finding is in accordance with modern concepts of the mechanism of proteinuria from which it would appear unlikely that any kidney should allow the escape of albumin and yet retain another protein so similar in size.

Summary

The addition *in vitro* of ^{59}Fe to the urine of patients suffering from the nephrotic syndrome and congestive cardiac failure, followed by paper electrophoresis, has been used to demonstrate the presence of transferrin, the iron-binding globulin.

A similar addition made to serum demonstrates the existence of an iron saturation limit and also the specific attachment of iron to transferrin.

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A SIMPLE METHOD FOR THE CONTINUOUS ELECTROPHORESIS OF SERUM PROTEINS

BY

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(RECEIVED FOR PUBLICATION APRIL 12, 1955)

In 1951 Durrum introduced a simple apparatus for the continuous separation of amino-acids, proteins, and other charged substances. Since then various modifications have been made based on the original Durrum idea to use a sheet of filter paper as the supporting medium. Most of these modifications have tended to make the construction and working of the apparatus much more difficult and therefore it was decided to attempt to construct the simplest possible apparatus for the separation of serum proteins. It was found that using the Durrum apparatus with a very few modifications the separation of serum proteins could be continuous for a period of one week.

The Apparatus

The apparatus is constructed as shown in Fig. 1 from ¼ in. perspex, and consists of a box 20 in. high, 14 in. wide, and 5 in. deep, with a detachable two-panelled front, the bottom panel 6 in. high and the top panel 14 in. high, having an upper buffer trough from which the paper curtain hangs and two lower subdivided electrode compartments into which the sides of the paper dip. The veronal buffer is continuously fed into the trough from a higher reservoir at a rate of 15 drops per minute and the excess flows by means of a constant levelling device into a small reservoir of approximate volume 20 ml., which, when full, flushes into the front lower buffer tanks. These tanks are subdivided so that the front compartment into which the paper dips is connected via two small holes to the back compartment into which the electrode vessel enters.

The electrode and lower buffer tank are shown in Fig. 2.

The most satisfactory paper so far used is Whatman No. 3 MM, cut with the machine direction of flow towards the anode; the dimensions of the curtain are shown in Fig. 3. The filter paper is folded so that 3.5 cm. of the paper folds back into the buffer trough and the rest of the paper hangs vertically as a curtain with 14 collection points at its base and the two outside tabs dripping into the two front buffer tanks. To increase the downward flow of buffer on either side of the paper one extra piece of paper is placed in each top corner, while three extra pieces are placed in the two bottom corners to increase the current flow across the base of the paper. A small tab, 1 mm. wide, is cut in the position shown and bent forward. A wick of the same paper 1 mm. wide connects this tab with serum in a watch glass at a higher level. Phenol red is added to the serum to serve as an indicator, which, when the apparatus is running correctly, migrates ahead of the albumin fraction. Before the wick and tab are connected the paper is allowed to reach saturation and a constant current flow.

A stable current is supplied to give 17 mA and 500 volts. The electrodes are prepared from 2 mm. silver wire bent into the required coil to fit the electrode vessels and coated with silver chloride. For convenience, the silver before it is coated is pushed through a polythene stopper which fits loosely into the glass electrode vessel. The electrode is immersed in a solution of saturated potassium chloride and is separated from the buffer tank by means of an agar bridge. This electrode system has proved very satisfactory in operation, as it eliminates any possible *p*H changes reaching the paper curtain and keeps the current steady throughout the separation.

This apparatus has been in constant use for the past 18 months and has proved very satisfactory for the separation of approximately 7 ml. of serum into its component fractions over a period of one week, provided that the silver electrodes are changed over every 24 hours.

When the required amount of serum has been separated the paper curtain is removed and dried horizontally at a temperature sufficient to denature the residual proteins; the paper is then stained by any of the usual protein staining techniques. From the stained pattern on the paper it is very simple to identify the protein fractions which have been collected into the various tubes, and we have found a remarkable consistency in the position of the protein fractions of various separations.

Reagents

Barbitone Buffer, pH 8.6.—Sodium diethylbarbiturate, 7.75 g., and 1.38 g. of diethylbarbituric acid are diluted to 1 litre with distilled water.

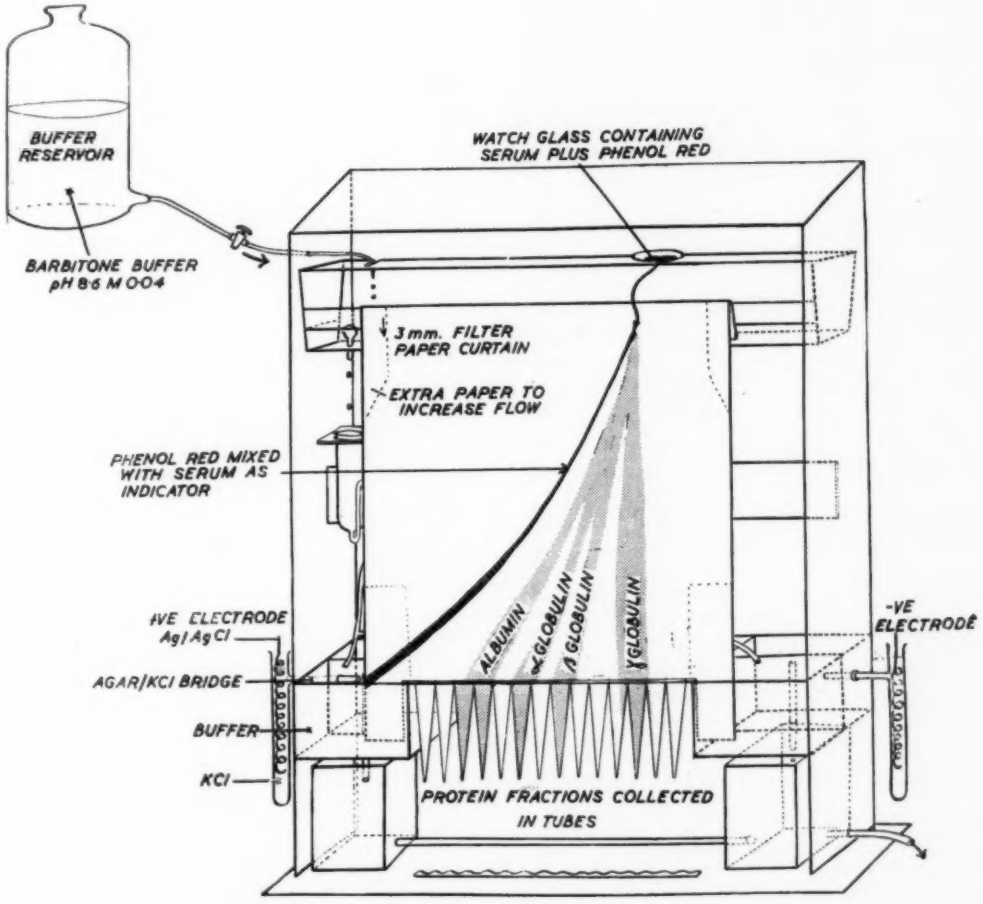

FIG. 1.—Diagram of apparatus.

FIG. 2.—Diagram of electrode and lower buffer tank.

FIG. 3.—Diagram of paper curtain.

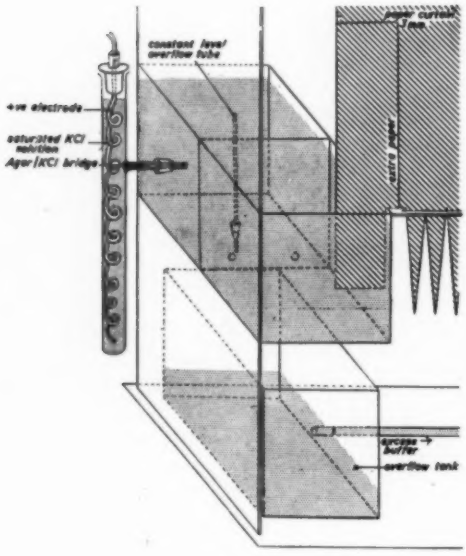

FIG. 2

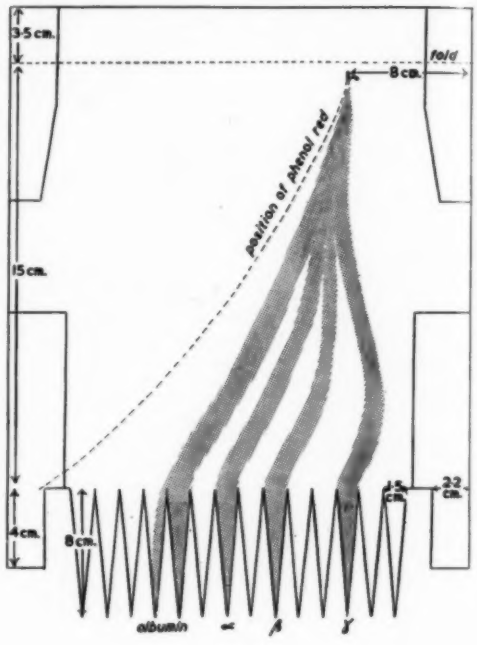

FIG. 3

Agar.—Agar, 2 g., is diluted to 100 ml. with a saturated solution of potassium chloride.

Preparation of the Silver/Silver Chloride Electrodes

One foot of 2 mm. silver wire is coiled into the required shape (to fit the electrode vessel) and first cleaned for a few seconds only in concentrated nitric acid and washed with distilled water. The silver wire is then placed as the anode in an electrolytic cell with a platinum cathode in 6% hydrochloric acid and a current of 20 mA is passed for 48 hours.

We wish to thank Professor Sir Charles Dodds for his interest in this work.

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TECHNICAL METHODS

A METHOD OF CONCENTRATING SPERMATOZOA IN HUMAN SEMEN

BY

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(RECEIVED FOR PUBLICATION APRIL 18, 1955)

When carrying out artificial insemination it is inconvenient to use more than 1 ml. of semen in most cases. When the husband's semen is oligospermic, or has a low percentage of motile sperm, it is an advantage if a high proportion of the sperm content can be concentrated in a small volume of the fluid. Even when the number and motility of the spermatozoa are satisfactory, if some hours must elapse between ejaculation and insemination the viability of the sperm may be improved by the addition of suitable diluents, as is the usual practice in animal insemination. In human semen, however, the improved motility due to dilution is seldom enough to compensate for the reduction in density, unless the sperm are subsequently concentrated by centrifuging or some other means. The centrifugation of human semen, while it concentrates the sperm, frequently depresses the motility, inevitably concentrates also debris and mucus, and, in the case of very mucoid specimens, may be quite useless at speeds which do not injure the active cells. A much easier method of obtaining a high proportion of the sperm content of the ejaculate in a small volume is to ask the donor to collect the first part of the ejaculate in a separate container from the remainder.

The higher sperm content and greater motility in the first part of partitioned ejaculates have been reported by MacLeod and Hotchkiss (1942) and more recently by Oettle (1954). Lundquist (1949) has investigated the biochemistry of the different fractions, and we are employing his results in a study of the pattern of ejaculation and the activity of the accessory glands in husbands of sub-fertile marriages. However, the collection of semen in separate fractions has proved so useful for A.I.H. (homologous artificial insemination) that it seemed worth while to give a preliminary report on this application of the work.

The following report is based on 93 specimens from 21 donors, 15 of whom were sub-fertile with a "fertility index" (Harvey, 1953) of less than 30, and six having potentially fertile semen. In each case the density and motility of the spermatozoa in both fractions of the ejaculate were determined by the usual methods (Harvey and Jackson, 1945) and the corresponding values which would have been found had the whole specimen been collected in one container were calculated. In what follows statements about the whole specimen refer to these calculated values.

Method

The relative concentration of sperm in the first fraction is conveniently measured by the ratio of the density of this fraction to that of the whole ejaculate. Thus a semen having a sperm density of 10m./ml. in the complete ejaculate but of 35m./ml. in the first fraction would give a ratio of 3.5, which means that it would have been necessary to use 3.5 ml. of the undivided ejaculate for insemination in order to introduce as many sperm as are present in 1 ml. of the first fraction. If the volume of the first fraction is less than 1 ml. the total number of sperm in the first fraction must be substituted for its density. For instance, if in the above example only 0.6 ml. of fluid was collected in the first container, the total sperm content of this fraction would be 0.6 × 35m., and only 21m. sperm would be introduced into the cervix if the whole of the 0.6 ml. was used for insemination. For practical purposes therefore the

TABLE I
FREQUENCY OF VARIOUS DEGREES OF CONCENTRATION OF SPERM IN FIRST FRACTION

	No. of Ejaculates	Ratio of Sperm in 1 ml. of First Fraction of Ejaculate (or Whole Fraction if Less than 1 ml.) to Sperm in 1 ml. of Complete Ejaculate				
		0·8 First Fraction Inferior	0·8–1·2 No Significant Difference	1·21–1·99 Fair Concentration	2·0 and Over Good Concentration	Highest Value
All sperm	93	8 (8·6%)	16 (17·2%)	48 (51·6%)	21 (22·6%)	3·9
Motile sperm	87	3 (3·5%)	4 (4·6%)	41 (47·1%)	39 (44·8%)	4·6

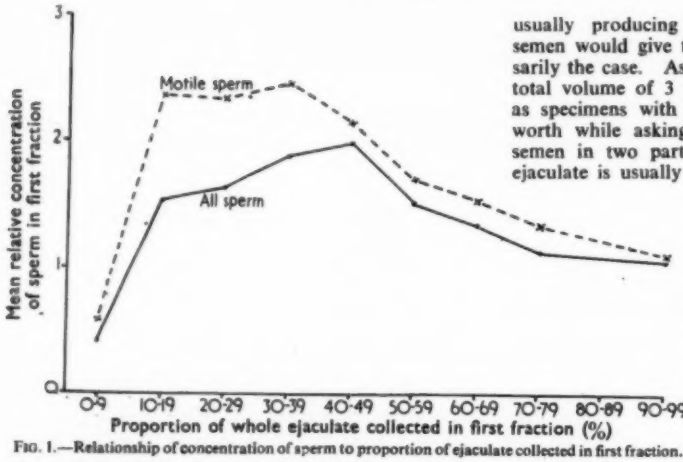

FIG. 1.—Relationship of concentration of sperm to proportion of ejaculate collected in first fraction.

improvement in this case, compared with 1 ml. of the complete ejaculate, would be only 2.1, not 3.5.

The density of motile sperm in the first fraction may be compared with the number in the whole ejaculate in the same way, the actual number of motile sperm in 1 ml. of fluid being of more interest than the percentage motility.

The sperm content of the first fraction may be regarded as significantly higher than that of the whole specimen if the ratio obtained in this way is greater than 1.2, and significantly less if the ratio is less than 0.8. The degree of concentration obtained in this series is shown in Table I.

Table II and Fig. 1 show the variation in the mean value of the "first fraction/whole specimen" ratio with different proportions of the ejaculate in the first fraction. The superior motility of the earlier part of the ejaculate, which is one important reason for using this fraction for insemination, is clearly shown in the graph.

Differences in the efficiency of concentration occur with different donors, and with the same donor on different occasions. It might be expected that men usually producing exceptionally large volumes of semen would give the best results; this is not necessarily the case. As shown in Table III, semen with a total volume of 3 to 4 ml. may concentrate as well as specimens with larger volumes. It is obviously worth while asking an A.I.H. donor to collect his semen in two parts if the volume of his complete ejaculate is usually over 2 ml.

It does not necessarily follow that the chances of conception following insemination are appreciably improved by using the first fraction of the ejaculate. Where the total sperm count of the whole ejaculate is only 1 or 2 million, or where the motility is very low, putting all the sperm available into 1 ml. of fluid does does not greatly increase the chance of success. If two "fertility indices" are calculated for each divided ejaculate, one based on the first fraction and the other on the complete specimen, in some cases the former is appreciably higher than the latter, while in other cases there is very little difference between the two. Successes have so far occurred only with semen that has given a higher value for the "fertility index" when based on the first fraction, but it is too early to say whether such a difference in the index is a reliable criterion of the usefulness of dividing the ejaculate in the case of any given donor. There is, however, nothing to be lost by collecting semen in this way. Pregnancies resulting from use of the first fraction have shown that the absence of the plasma of the second part of the ejaculate does not impair the fertility of the sperm in the first part. In the rare cases where the first fraction is inferior, or insufficient in amount, the second fraction will be similar to the undivided ejaculate, and may be used for insemination instead of, or mixed with, the first fraction.

TABLE II

RELATION OF SPERM CONCENTRATION IN FIRST FRACTION TO PROPORTION OF EJACULATE COLLECTED

% of Ejaculate Collected in First Fraction	Mean Volume of First Fraction (ml.)	Concentration of Sperm in First Fraction			
		All Sperm		Motile Sperm	
		No. of Ejaculates	Mean Relative Concentration	Ejaculates	Mean Relative Concentration
0–9	0·35	2	0·42	2	0·58
10–19	0·52	5	1·52	5	2·36
20–29	1·14	18	1·63	17	2·34
30–39	1·47	15	1·89	14	2·45
40–49	1·48	17	1·98	15	2·15
50–59	2·45	18	1·51	17	1·7
60–69	2·01	9	1·35	9	1·54
70–79	2·31	8	1·23	7	1·35
80–89	—	—	—	—	—
90–	4·5	1	1·07	1	1·12

TABLE III

RELATION OF CONCENTRATION OF SPERM IN FIRST FRACTION TO VOLUME OF EJACULATE

Vol. of Whole Ejaculate (ml.)	Concentration of Sperm in First Fraction			
	All Sperm		Motile Sperm	
	Ejaculates	Mean Relative Concentration	Ejaculates	Mean Relative Concentration
1–1·9	7	1·3	7	1·43
2–2·9	26	1·55	23	1·9
3–3·9	21	1·72	20	2·09
4–4·9	13	1·6	12	2·05
5–5·9	7	1·85	7	2·35
6–6·9	6	1·52	6	2·04
7–7·9	7	1·28	7	2·2
8–9·9	6	2·04	6	2·5

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J. clin. Path. (1955), 8, 343.

THE STAINING OF BACTERIAL CAPSULES AND SLIME WITH FERRIC COMPOUNDS

BY

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In 1946 Hale described a method for demonstrating acid mucopolysaccharides in sections based on the affinity of these compounds for colloidal iron oxide in acid solution. The combined iron was demonstrated by conversion to prussian blue.

It has been found that bacterial capsules and slime are very well stained by Hale's method. This is possibly due to their content of acid mucopolysaccharides, but this is not certain, since doubts have been cast on the specificity of this reaction (Pearse, 1953).

The method of Rinehart and Abul-Haj (1951) is also applicable, but the iron solution is troublesome to prepare and the results are no better than with Hale's method.

The following technique is based on Hale's method, but saccharated iron oxide is used, resulting in considerably deeper staining.

Method

(1) Thin films of saline suspensions are dried and fixed by heat.

(2) Equal volumes of "ferrivenin" (Benger) and 2 M acetic acid are mixed, centrifuged, and the slide is flooded with the clear supernate. It is then left for one to 10 minutes.

(3) The slide is washed well in distilled water.

(4) It is flooded with a mixture of equal volumes of 0.14 M hydrochloric acid and 0.02 M potassium ferrocyanide and left for 10 minutes.

(5) It is washed in distilled water.

(6) If desired it is counterstained in 1% neutral red.

Results

Capsules and slime stain deep blue (Figs. 1 and 2). Thick capsules may be almost black, while thin slime layers are more often greenish blue.

The great majority of bacteria show a peripheral blue-stained layer, more marked when the organisms are in a mucoid phase.

Some organisms, e.g., *Staphylococcus citreus*, may be stained throughout, with deeper blue rims.

Many bacilli, e.g., *Salmonella, B. proteus, Ps. pyocyanea, Cl. welchii*, show a bipolar concentration of blue-stained material.

Bacilli containing spores, e.g., *B. cereus, Cl. sporogenes*, show deep, uniform staining of the bacillary body, the spore being unstained.

Notes

Once acidified, the colloidal iron solutions are unstable and slowly throw down a precipitate. The solutions must therefore be centrifuged or

FIG. 1.—*Str. pneumoniae* Type III. Saccharated iron oxide, × approximately 2,500.

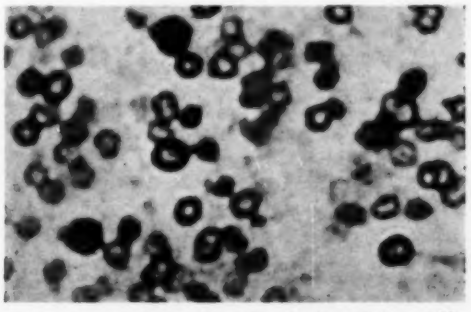

FIG. 2.—*Str. pyogenes* Group C. Saccharated iron oxide, × approximately 2,500.

filtered on the day of use if they are kept for more than one day. Acidified "ferrivenin" has given unimpaired results after keeping for three months, but Hale's and Rinehart and Abul-Haj's stains give progressively weaker staining on storage and it is best to add the acid just before use.

A dextran-saccharated iron oxide complex and another preparation of saccharated iron oxide available commercially gave very weak staining.

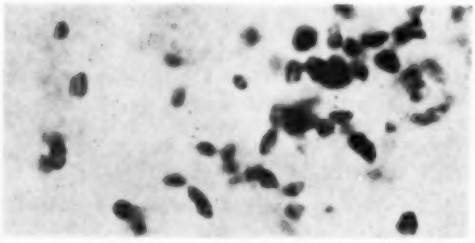

FIG. 3.—Klebsiella in mouse spleen. Paraffin section. Hale's method. × 4,750.

Potassium ferrocyanide solution should be stored in the dark to delay the yellowing which it slowly undergoes and which is accompanied by a weakening of the final stain.

If bacterial capsules are to be stained in paraffin sections Hale's method should be used (Fig. 3). Saccharated iron oxide stains other tissue components too deeply to be used for this purpose.

Slime is stained more intensely if ammonium sulphide is substituted for acidified ferrocyanide. The results are not permanent, however.

I wish to thank Dr. H. Loewenthal, Senior Pathologist at Chase Farm Hospital, for his interest and help in this work. Mr. H. Knight took the photographs.

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J. clin. Path. (1955), **8**, 345.

THE APPLICATION OF RADIANT HEATING IN THE CLINICAL LABORATORY

BY

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(RECEIVED FOR PUBLICATION OCTOBER 12, 1954)

Since Priestley utilized the radiant energy of the sun with his burning glass in his famous experiment of 1774, the method has fallen into disuse and the chemist has accepted the water-bath, the hot-air oven, and the furnace as the standard tools for evaporation, drying, and combustion.

The boiling water-bath is used for evaporating solutions or drying solids which decompose at temperatures slightly above 100° C. It is slow; it wastes nearly all of the energy used; it requires a constant supply of cold water and a constant overflow into a sink; and steam condenses on every available cold surface and helps metals to corrode or wood to rot. If it is used for evaporating volatile solvents, they readily superheat so that loss is often caused by bumping. If gas heating is used for evaporating inflammable solvents, there is a serious hazard of fire, while electrically heated baths have a fairly short life and are expensive.

Drying ovens, often jacketed by steam or toluene vapour, are inefficient because they depend on conduction and convection to transfer heat from the walls of the chamber to an object which is maintained at almost the same temperature. Provision for the removal of vapour is rarely adequate, and the ovens corrode rapidly.

Many of the objections to the drying oven apply to the furnace, but since the furnace is usually well lagged it is more efficient. The main disadvantages are its bulk, high cost, and high energy consumption.

Radiant energy is widely used in industry. It travels in straight lines and is converted into heat when it strikes and is absorbed by an object. We have used it to evaporate to dryness aqueous solutions; to evaporate inflammable solvents; to dry solids without overheating; and to ash stools and other organic material. We have found that our methods employing radiant energy are cheaper, quicker, and more convenient than the conventional methods. In particular, in dealing with inflammable solvents, the hazard of fire is obviated and the danger of loss by bumping is eliminated.

Technique

The source of radiant energy is the G.E.C. 250-watt infra-red reflector lamp, which includes an untarnishable reflector and produces a roughly parallel beam of radiant energy with maximum intensity at a wavelength of about 1.2 μ. Ordinary glass is transparent to this radiation, and polished metals reflect at least three-quarters of it. In order to make maximum use of the energy for evaporating solvents, heat-absorbing bodies must be placed inside the vessel containing the solvent, and material to be ashed should be placed in a tarnished crucible made of a metal such as nickel. This lamp is screwed into its holder and held in a clamp so that it points vertically downwards on to a sheet of asbestos.

Evaporation of Urine and Aqueous Solutions.—The liquid is placed in a nickel dish, or, with less efficiency, in a porcelain dish. The lamp is directed vertically on to the surface from a distance of about 6 in., and the liquid evaporates quietly, 25 ml. of water requiring about 30 minutes' exposure.

Drying of Stools and Similar Solids.—This can be done in a manner similar to the evaporation of urine, the solid being spread out in a thin layer to keep the surface at a maximum. It is better to spread the stool over a porcelain boat which is slid into a wide glass tube through which a slow stream of air is drawn, removing water vapour and smells, and thus making a fume cupboard unnecessary. A standard "quickfit" socket, SRB29, on a tube 140 mm. long and of 30 mm. lumen is suitable. Suction can be applied through a "quickfit" cone to rubber tubing adaptor MF10/4 (B29 cone to straight glass tube). We use a Royal Worcester porcelain drying boat 100 mm. long, 18 mm. wide, and 10 mm. deep. A rectangular piece of polished aluminium, 9 × 6 in., curved about its short axis so that it forms part of a cylinder with a radius of about 4 in., is placed on the bench beneath the tube so that the tube lies along the axis of the partial cylinder. This serves to protect the bench and to reflect on to the tube energy which would otherwise be lost (Fig. 1).

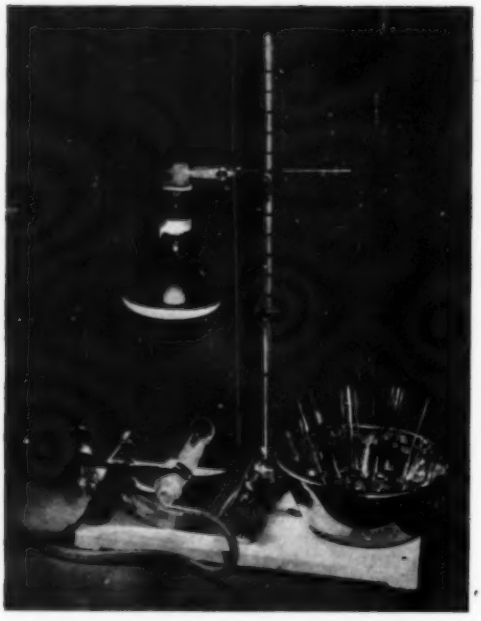

FIG. 1.—Apparatus for evaporating liquids and for drying solids. The lamp is clamped to a rod graduated in inches and is readily moved in vertical and horizontal planes. The bowl is not fixed and can be replaced by an evaporating dish or several small crucibles.

A sample of stool sufficient for the estimation of fat by the method of Holt, Courtenay, and Fales (1919) can be dried in 10 to 30 minutes, depending on its consistency.

Evaporation of Inflammable Solvents.—A stainless steel bowl, 8 in. in diameter, is fitted internally with 10 Terry clips to hold 6 × 1-in. boiling-tubes. A piece of stainless steel or aluminium sheet is cut and bent into the form of a right circular cone 3 in. in diameter and riveted in that position, then being placed on its base in the centre of the bottom of the bowl. The solutions to be evaporated are placed in the boiling tubes and two or three black glass beads added to absorb the radiant energy. The lamp is placed 2 to 4 in. above the rim on the centre line of the bowl. Quiet boiling begins in a few seconds, and 10 samples, each of 20 ml. of alcohol-ether mixture, can be evaporated to dryness in about half an hour without any risk of fire or bumping (Fig. 1).

Distillation.—When radiant energy is used, the transfer of heat energy to a liquid can be controlled instantaneously by the turn of a switch. If the liquid to be distilled is transparent to radiant energy in the 1–2 μ band, the distilling flask must contain beads which absorb this radiation. There is no fire risk, and since glass is transparent to this wavelength the apparatus and its surroundings are never heated above the boiling point of the liquid.

Ashing of Organic Material.—If the radiant energy from an infra-red lamp is directed axially into a parabolic reflector obtained from a large automobile headlight, it is concentrated at the focal point of the mirror and an absorbing object placed there becomes very hot.

The apparatus used consists of a nickel-plated copper sleeve and flange which fits into the recess at the periphery of the reflector. A nickel crucible is held on a wire ring fixed to a brass rod which passes through the aperture at the centre of the reflector. Lead chloride placed inside the crucible melts in about two minutes (temperature 501° C.), lithium carbonate fuses in about 10 minutes (temperature 618° C.), and the maximum attainable temperature with our apparatus is about 650° C., which is reached in 15 to 20 minutes. One gram of dried faeces yields a pale grey ash in 20 to 45 minutes, according to the fat content.

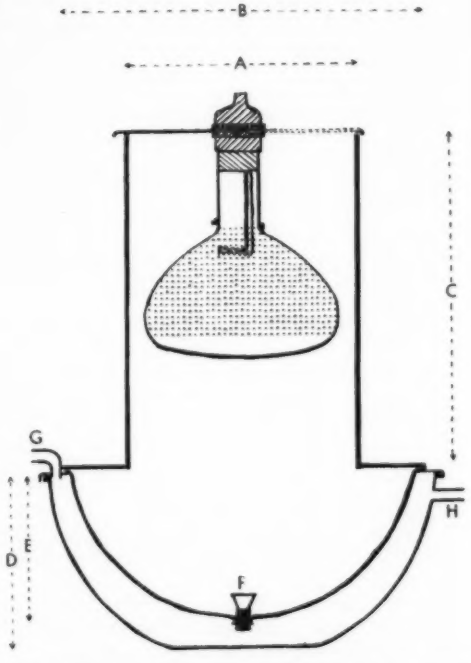

FIG. 2.—Micro-furnace. The lamp holder is fixed in a star support which rests on the support and shield AC—A 5½ in., C 9 in. The reflector is 9 in. diameter and 3½ in. deep. D is a steel bowl 11½ in. diameter and 4½ in. deep. G and H are the inflow and the outflow for the cooling water. F, a wire ring support for the crucible, is carried on a rod which is silver-soldered into the aperture of the reflector, and seals the aperture hermetically. The tube G is soldered into a brass annulus which is riveted to the reflector flange and turned down over the edge of the stainless steel bowl. The only joints which need be watertight are those at F and H.

Since some energy is absorbed by the reflector, after prolonged use it becomes very hot, and tends to damage the plating. This difficulty was readily overcome by immersing the reflector in a bowl of flowing cold water. The apparatus* is illustrated in Fig. 2.

Conclusions

These infra-red lamps cost about 15s. and have a life of more than 5,000 hours. Ancillary apparatus is simple and cheap. The automobile headlamp reflector was obtained from a car breaker's yard and was cleaned and nickel plated. They allow one to evaporate or distil inflammable solvents without hazard of fire and without mess, and can replace the muffle furnace in many metabolic studies. Even small laboratories can consider undertaking sodium and potassium balances. After a six months' trial of infra-red lamps we are using boiling-water baths only to provide a standard temperature for the development of colours in colorimetric analysis and to hasten the rate of chemical reactions. If standard glassware could be made from infra-red absorbing glass, the radiant heat lamp could become even more useful. Discussions with some glass manufacturers are now in progress.

* An improved version is being manufactured by Messrs. Townson and Mercer, Croydon.

Summary

Infra-red lamps may be used with advantage to (1) evaporate liquids, especially inflammable liquids, (2) dry solids, (3) combust organic material.

Infra-red lamps are essentially clean and lose little heat to their surroundings. Methods are described for evaporation, drying, and incineration which are more efficient and cheaper than conventional methods.

I am glad to express my thanks to Dr. George Discombe for his help and encouragement, and to Mr. F. Dewar for his help in making the apparatus.

References

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ASSOCIATION OF CLINICAL PATHOLOGISTS: 55th GENERAL MEETING

The 55th general meeting of the Association of Clinical Pathologists was held at the Royal College of Surgeons, Lincoln's Inn Fields, London, from September 29 to October 1, 1955.

The scientific sessions included one devoted to a joint meeting of the Association of Clinical Pathologists and the Association of Clinical Biochemists.

Two formal lectures were delivered, the Panton Memorial Lecture by Professor S. P. Bedson on "Viral Multiplication and the Chemotherapy of Viral Infections," and the Foundation Lecture by Dr. J. G. Greenfield on "The Clinical Pathologist and Encephalomyelitis: Past, Present, and Future." It is hoped to publish both these lectures in full.

Demonstrations were on view for two days, and a new feature was a histopathological quiz, in which Dr. Stewart Smith, of Exeter, conducted a novel experiment by producing four well-documented cases with unusual features in their histological biopsy specimens. Members were invited to fill up a form with their diagnosis and comments. The experiment undoubtedly proved successful, and it is hoped that it will be repeated at future meetings.

The Pathology of Fatal Aircraft Accidents

J. K. MASON said that until recent years the limitations upon airframe and engine structure had been such as to confine the human factors in aviation to the sphere of the physiologist. The introduction of the jet engine had increased the hazards of flight to the extent that aviation pathology has emerged as an autonomous subject with the following objectives.

(*a*) To correlate the post-mortem findings in victims of fatal aircraft accidents with engineering and other evidence, thus helping to elucidate obscure causes of accidents. (*b*) To bring to light any recurring injuries which may suggest faults in aircraft design. (*c*) To apply post-mortem appearances to the assessment of new and experimental types of safety equipment. (*d*) To search for pre-existing disease in aircrews which might be missed on ordinary methods of medical examination and yet have a bearing on aircraft accidents. (*e*) To clarify some of the confusion which surrounds traumatic pathology in general and aviation pathology in particular. Such uncertain aspects of aviation pathology include the pathology of anoxia, injuries induced by pure accelerative and decelerative forces, the aetiology and prognosis of fat embolism, and, most importantly, the pathology of explosive decompression.

The Pathology of the Comet Aircraft Disasters

D. I. FRYER (R.A.F. Institute of Aviation Medicine, Farnborough) reported that from the Comet aircraft which crashed at Elba in January, 1954, and near Naples in April, 1954, 20 bodies were recovered.

The immediate conclusion which could be drawn from the examination of the bodies was that the occupants of the aircraft had been subjected to two phases of violence: an upward and forward displacement within the cabin, and an abrupt deceleration on impact with the sea, following free-fall from altitude. The skeletal injuries received in the latter phase appeared to be post-mortem in type. However, the amount of bleeding from visceral injuries made it difficult to decide whether these had occurred during the first or second phases, and, also, their severity was such that their origins were obscure.

Experiments revealed that the abdominal and thoracic lesions could have resulted from impact with the water, and that the degree of haemorrhage was not incompatible with the theory that the first phase of the accident had already caused fatal injury. Structural, physiological, and pathological considerations excluded explosive decompression as the sole or major cause of the lung injury observed.

Two mysterious types of skin lesion observed were found to be due to scalding by the surface layer of the sea beneath a film of burning kerosene, and post-mortem melanin pigment darkening as a response to the long-wave ultra-violet fraction of sunlight.

Some Histopathology of the Skin

JOHN O. OLIVER (St. John's Hospital for Diseases of the Skin, London), after reviewing briefly the record of biopsies at St. John's Hospital for Diseases of the Skin, over a period of one year, listed those diagnoses which had been made more than 12 times during the year. After defining some of the special terms used in dermatology, Dr. Oliver considered briefly the question of the skin biopsy and its subsequent handling in the technical laboratory, urging the inclusion of normal skin in a biopsy of any skin lesion. The subjects chosen for histological description by means of colour photomicrographs were eczema (with lichen simplex), intra-epidermal epithelioma of Borst-Jadassohn, granuloma annulare, histiocytomas (sclerosing haemangiomas), *Lichen planus*, lupus erythematosus, and molluscum sebaceum (kerato-acanthoma).

Radioactive Iron Techniques in Routine Haematological Practice

G. WETHERLEY-MEIN, M. S. R. HUTT, W. A. LANGMEAD, and M. J. HILL (St. Thomas's Hospital, London) described the techniques used in iron tracer studies with radioactive iron, Fe 59.

The iron is injected intravenously either bound to β globulin or after addition to 1% sodium citrate in saline. Three methods are used to determine how the body deals with the injected dose.

First, it is possible to measure the rate at which it is cleared from the plasma. Samples of plasma are withdrawn at intervals during the first hour and a half after injection and counted in the sample counter. The plasma is cleared exponentially so that it is possible to calculate the half-clearance time. The results are usually what might be expected, in that patients with marrow hypoplasia or aplasia have prolonged half-clearance times, while in polycythaemia vera, haemolytic anaemia, and iron-deficiency anaemia the iron was removed from the plasma very rapidly. The method, however, has considerable limitations. First, it only measures clearance from the plasma and does not show whether the iron is stored or whether it is used for erythropoiesis. Secondly, because of variations in circulation and mixing rates, the normal range is very wide and therefore a clear-cut answer is only possible in extreme cases. Thirdly, it involves the patient in at least five venepunctures in just over an hour.

It is possible to get a better idea of how the body is using iron by using a surface counting technique. Following injection of iron its movement in the body can be followed by applying a directional counter and recording changes in counting rates over the heart (a measure of blood activity), the spleen, the sacrum (a measure of marrow activity), and the liver. In the normal patient there is an initial fall in the heart count associated with a rise in the marrow count as iron is taken up by the marrow. This reaches its peak at about 30 hours and then falls as red cells containing active iron are released into the circulation and this is associated with a secondary rise in the heart count. By contrast patients with aplastic anaemia show no measurable uptake of iron by the marrow, the heart count declines slowly, and a steadily increasing activity over the liver site indicates storage of iron in this organ.

Thirdly, by measuring the activity of samples of red cells on the seventh post-injection day an estimate of the percentage of the injected dose appearing in red cells can be obtained. The percentage utilization depends first on the iron stores of the body and secondly on the red cell turnover. A high percentage utilization in a patient with normal iron stores implies a rapid marrow turnover as in haemolytic anaemia. A low percentage utilization implies either increased iron stores as in haemochromatosis, or a poor red cell turnover as in aplastic and hypoplastic anaemias.

SYMPOSIUM: THE COMPLICATIONS AND FAILURES OF DRUG THERAPY

Professor M. L. Rosenheim opened the symposium.

Bacteriological Aspects of Failures of Drug Therapy

R. KNOX (Guy's Hospital) stated that the problem in drug treatment of infections might be summed up as one of attacking at the right time and in the right place the right organism with the right drug. First, time; the organisms may not be in the right phase of growth. Dormant organisms are much less vulnerable than rapidly growing organisms. Secondly, situation; in a chronic lesion chemotherapy is much less likely to be successful than in a fulminating septicaemia. Accessibility is important, and therefore knowledge of how a drug is absorbed is essential. Thirdly, the wrong drug or the wrong organism; there are several ways in which this may occur. (1) Mistakes in the laboratory—deterioration of drugs, wrong identification of organisms, etc. (2) Drug resistance which may either develop in the course of treating a particular patient or may be the result of cross-infection with resistant strains. "Super-infections" with drug-resistant fungi or bacteria must be mentioned. (3) Wrong interpretation or misleading results. There is great variability in sensitivity tests as commonly performed; perhaps the time has come for an attempt to achieve some uniformity in clinical laboratories. The tests themselves also have inherent weaknesses. They tell little about the distribution of resistant organisms in a bacterial population, whether the drug is bactericidal or bacteriostatic and little about the possible value of different combinations of drugs. A particular combination may be highly bactericidal, though the individual drugs are not.

Complications and Failures in Antibiotic Treatment

K. B. ROGERS (Birmingham Children's Hospital) said that some of the failures and complications of antibiotic therapy could be anticipated, but often one was confronted by an unpredicted reaction by an individual patient, or a failure due to a particular condition that prevented an antibiotic acting. The most common complications of antibiotic therapy are due to cross-infection by insensitive organisms such as staphylococci and moniliasis of the lungs or intestines. If there is fibrosis and loculation, as in pericarditis, empyema, or brain abscess, a theoretically effective antibiotic may not penetrate to the infecting organisms. A patient may develop a pyrexia during prolonged penicillin or streptomycin therapy. It may be difficult to prove that the temperature is solely raised because of the antibiotic, and it requires a very fine clinical judgment to discontinue therapy. Allergic manifestations to penicillin and streptomycin are relatively common, such as cutaneous hypersensitivity amongst those who handle them constantly, local irritation at the site of inoculation, and relatively rarely anaphylactoid reactions to penicillin.

Four cases of anuria, which were probaby due to penicillin therapy, had been encountered. The anuria lasted from four to eight days in children between the ages of 8 months and 5 years. All recovered after treatment with Bull's regime, although one has a permanent disability after a further complication of monilial cystitis.

Uncontrolled prophylactic " umbrella " therapy has produced staphylococci resistant to a wide range of antibiotics, and these have caused serious complications in surgical units, such as chest centres. This can be overcome if the surgeons and the laboratory co-operate.

Haematological Aspects of Toxic Reactions

WILLIAM M. DAVIDSON (King's College Hospital, London) said that the use of certain drugs was at present surrounded by fear of unpredictable toxic consequences. The fear was partly real and partly exaggerated, owing to emphasis on the disasters without appreciation of their frequency.

The toxic reactions extend from eosinophilia, mild anaemia, or leucopenia, through the more serious acute haemolytic anaemia, thrombocytopenic purpura, and agranulocytic angina, to the dreaded marrow destruction of aplastic anaemia.

The present method of counting the white cells at intervals or of advising the patient to stop the drug if any symptoms arise is so far from the ideal as to be really useless. The problem does not concern the very poisonous drugs which merely cause toxic reactions because of over-dosage, but those where doses recognized as safe may cause reactions in certain individuals.

Evidence has been produced that some of these drugs, such as amidopyrine, sedormid, and phenacetin, act directly upon and destroy the circulating cells or platelets. The method of destruction of the cells is often akin to the ordinary immune reaction in the presence of complement, and it seems that the drug forms a complex with the cell protein which, being both antigenic and foreign to the individual, excites an antibody–antigen reaction. In other cases there may be an inherent defect in the cells in certain individuals which make them unduly sensitive, as has been demonstrated in " primiquine " haemolysis. Again an abnormal pathway may lead to the formation of abnormal potentially toxic metabolites. Other drugs would seem to act indirectly through the precursor cells in the marrow. Either the marrow is stimulated to produce excessive numbers of leucocytes or depressed up to the point of destruction, with, after an appropriate interval, progressive disappearance of the circulating elements. In such cases there may be some mechanism whereby an abnormally high concentration of the drug or its metabolites is achieved in the marrow, and figures suggesting a 20-fold concentration, as compared with the serum, have been reported. In some cases a type of Arthus phenomenon may develop in and destroy the marrow. These various mechanisms explain to some extent the differences in the reactions induced by various drugs, and particularly the distressing fact that bone-marrow damage may not become obvious till weeks after the treatment ceased.

Recently work on " primiquine," an antimalarial drug, has turned attention to a new facet of the problem, the possibility of detecting, *in vitro*, red cells which will be sensitive. Further development along these lines might be helpful.

At the present stage it seems that more careful weighing of the real risk with each drug against the danger of the illness and the benefits expected, possibly the examination of the blood and marrow at critical times during the treatment, and above all the finding of less toxic but equally effective drugs offer the best chance to reduce the present, fortunately very small, but nevertheless disturbing, death rate from therapy.

Antibiotic Combinations for the Suppression of Resistant Variants in Urinary Infections

H. STERN and S. D. ELEK (St. George's Hospital, London) said that combinations of streptomycin, chloramphenicol, and tetracycline had been tested, using the gradient plate technique, for their value in suppressing the emergence of resistant variants of coliform bacilli from cases of urinary infection.

Streptomycin plus chloramphenicol or tetracycline are the most effective combinations. Not only are resistant variants suppressed but these combinations possess bactericidal activity which is probably an important factor in preventing relapses in urinary infections. Chloramphenicol plus tetracycline prevent the emergence of resistant variants but have little or no bactericidal action against most strains.

Calculations of the chances of a variant arising which is simultaneously resistant to two antibiotics show that the efficacy of combined treatment is explained by the large size of the bacterial population required for such doubly resistant variants to emerge. It is unlikely that during the course of an acute urinary infection sufficiently large bacterial populations can be attained.

It is suggested that these findings have an application in clinical practice.

The Use of the Laboratory in the Diagnosis and Management of Pituitary-Adrenal Syndromes

F. T. G. PRUNTY, R. V. BROOKS, and IVOR H. MILLS (St. Thomas's Hospital Medical School, London) reported that indirect methods of assessing adrenal cortical function had recently been reviewed (Prunty, 1950). The behaviour of the gland gives the most important laboratory information in these conditions. The most recent advances concern methods of measuring adrenal cortical secretions and their metabolites and further knowledge of abnormalities of electrolyte and water metabolism. The syndromes under dis-

cussion broadly divide themselves into normal or perverted overfunction and decreased activity. Both of these groups may be associated with change in pituitary activity; some information in this respect has been obtained by the difficult technique of A.C.T.H. assay in these patients. The modern use of elective surgery on adrenals and pituitary must not be overlooked.

According to current views the adrenal cortex primarily secretes 17 hydroxycorticosterone (F), corticosterone B, aldosterone, and 11-β-hydroxy androstenedione. Well-defined reaction patterns of corticoid metabolism may be discerned and 14 C.21 end-products are known in the urine.

Blood corticoid analyses have their own advantages, but even here only a small fraction is present as free and active hydrocortisone. As with urine metabolites the vexed question of relative amounts of "F" and "B" secreted by the adrenal is raised and also the problem of conjugates.

The application of these techniques to the diagnostic problems was briefly surveyed. Predominantly important is the diagnosis of Cushing's syndrome and the type of adrenal lesion associated with it. Doubt which remains may possibly perhaps be dispelled by the use of A.C.T.H., but special care over the variability of base-line urine and steroid levels is needed. Here eosinophil levels can be very helpful when due precautions are taken.

The method for determining 17-ketogenic steroids is very valuable but includes estimation of pregnanetriol, notably present in patients with the adrenogenital syndrome. This substance is easily estimated by the method of Bongiovanni and Clayton (1954). Its assay and that of 17-ketosteroids are of value in assessing treatment in this syndrome. The therapy of Cushing's syndrome and patients under treatment with A.C.T.H. can also be assessed with the methods discussed.

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Prunty, F. T. G. (1950). *Journal of Clinical Pathology*, **3**, 87.

The Determination and Significance of 17-Ketogenic Steroids in the Urine

ARTHUR JORDAN (Sheffield) said that Norymberski introduced the determination of the 17-ketogenic steroids into clinical medicine as an index of adrenal cortical activity; he showed that oxidation would convert some of the metabolites of corticoids into 17-ketosteroids. Over the age of 16, 80% of people have outputs of 17-ketogenic steroids ranging from 5.7 to 17.7 mg./day. The output increases with the administration of cortisone or A.C.T.H. The output tends to be low in Addison's disease and Simmonds's disease and high in Cushing's syndrome, in virilism, and in precocious puberty of adrenal cortical origin. Observation of the output following A.C.T.H. administration appears to indicate whether the adrenal cortex can respond and is a useful diagnostic procedure in suspected Addison's disease.

Adrenal Corticoid Excretion in Cushing's Syndrome

C. L. COPE (Postgraduate School, Hammersmith, London) said that isotope studies showed that hydrocortisone is rapidly metabolized into a number of compounds of which only a small fraction remain chloroform soluble. Chloroform extracts of urine will thus contain only a part of the adrenal metabolites, and this may not be a constant fraction of the total. Of the many chemical methods proposed for measuring adrenal activity some suffer from chemical unreliability, but others, though chemically valid, will estimate an empirical fraction of the total metabolites. Whilst most metabolites disappear almost completely from urine when adrenal function ceases, they do not necessarily all increase proportionally when adrenal function is stimulated.

Since hydrocortisone (F) is the natural product of the adrenal, its concentration is likely to reflect adrenal activity most closely. The behaviour of this steroid has been compared with that of tetrahydrocortisone (THE) and of tetrahydrohydrocortisone (THF). These have been estimated by separation on paper chromatograms and the use of blue tetrazolium.

In a series of nine cases of Cushing's syndrome the mean excretion of hydrocortisone was eight times the mean for normals, but THE excretion was only 1.8 times and THF only 2.7 times the normal mean.

Stimulation of three normal subjects with A.C.T.H. increased F excretion 7 times, but THE only twice and THF 2.5 times. In three cases of Cushing's syndrome similarly stimulated, F excretion was increased an average of nine times but THE and THF only 1.1 and 1.3 times respectively. Similarly, if Cushing's syndrome is inhibited by 9 fluorohydrocortisone a much greater reduction of F output than of the metabolites is achieved.

Changes in urinary F excretion reflect more rapidly and more sensitively variations in adrenal activity than do changes in such metabolites as THE or THF, which are comparatively rough indices.

The Significance of the Blood Acid and Alkaline Phosphatase Values in Cancer of the Prostate

STANLEY WRAY (Middlesbrough) said that the significance of the blood acid phosphatase value was assessed in 500 miscellaneous patients, 407 of whom had prostatic disease. It is shown that the formaldehyde-stable fraction is a better indication of the presence of cancer of the prostate than is the more usual investigation of the total value. It is also found that urinary retention may by itself cause a moderate increase in the blood acid phosphatase and that it must be relieved before reliable results can be obtained.

It is also shown that, when the blood alkaline phosphatase is estimated and graphically recorded in cases

of cancer of the prostate with bone secondaries on stilboestrol therapy, a definite pattern of response is obtained. This response pattern or alkaline phosphatase "kick" may be used in cases of prostatic cancer to (1) confirm diagnosis, (2) aid in doubtful cases, (3) help to assess the presence of osseous spread, (4) help in the choice of a therapeutic agent, and (5) act as "end-point" when assessing the value of therapy in other forms of cancer which give rise to bone secondaries.

The Effect of the Ingestion of Potassium Salts on the Excretion of Salt-retaining Corticoids in the Human Subject

H. ELLIS C. WILSON (Glasgow) reported that it had been shown in the normal subject that the plasma K falls after the ingestion of acid or alkaline K salts. It was suggested that tissue saturation and not plasma level controls the rate of K excretion (Wilson, 1948). The present investigations show an increase in the amount of salt-retaining corticoid in the urine (rat assay) after taking potassium salts. As the plasma K does not rise any direct effect on the pituitary or adrenal would be excluded. It is suggested that the K coming to the liver from the alimentary canal inhibits the breakdown of the corticoid. The increase in corticoid in the blood causes K excretion and a tendency to fall in plasma K which is made up by release of the ingested K from the liver. The hypothesis is put forward that the liver is one of the main factors controlling the amount of salt corticoid in circulation, adjusting the breakdown according to the amount of K exogenous or endogenous coming to it. The scheme suggested, along with the experimental results, shows that there is a quickly responsive mechanism for keeping the plasma K constant. This effect of K on the output of salt corticoid may be of significance in conditions where there is frequently a loss of K from the muscles associated with an increase in salt corticoid in the urine, such as congestive heart failure and nephrosis (Fox and Slobody, 1951). It is suggested that the release of K from the tissues may initiate its excretion by the mechanism proposed above.

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Aspects of Hypophysectomy

D. N. BARON (Royal Free Hospital, London) described the use of hypophysectomy and of radiation destruction of the hypophysis in the treatment of malignant disease, and the experience gained by the study of patients on whom the operation had been performed by Mr. E. J. Radley-Smith. Of 13 female patients with carcinoma of the breast who had been operated on within the past year, five had had a good result, with complete relief of pain and variable regression of the metastases. The rationale of the operation is the removal of somatotropin and luteotropin, as well as of A.C.T.H. and gonadotropins, for certain tumours of breast and prostate are dependent on hormones for their continued spread. It is too early to state whether hypophysectomy will replace adrenalectomy plus oöphorectomy in the treatment of carcinoma of the breast.

The tasks of the chemical pathologist in the preoperative assessment of patients (with particular regard to their adrenal function), in the biochemical control of the disturbances of metabolism, and in the difficult problem of assessing whether hypophysectomy has been complete, were discussed. Much further research is required on the latter problem, and on the aetiology of the different phases of water diuresis that follow the operation.

Blood Aceto-acetate Levels in the Immediate Post-operative Period

L. NAFTALIN (Lincoln) said that it had been shown by other workers that in the first post-operative days endogenous fat is burned at the rate of (approximately) 2,250 cal./day, i.e., many times the rate observed in semi-starvation. On the assumption that increased aceto-acetate is formed only when 2-C fragments are being produced in excess of immediate tissue needs serial examinations were made of blood aceto-acetate by the method of Walker (1954) in the hope of timing the switch-over to fat utilization. In evaluating the results account has to be taken of starvation ketosis and certain other variables; nevertheless, a sharp rise in the blood aceto-acetate level appears to occur regularly at an early stage after operative trauma, and sometimes after an anaesthetic (intravenous pentothal) only. The immediate rise is probably an adrenaline effect: the first high peak at four to five hours post-operatively may also be due to adrenaline, since A.C.T.H. and cortisone have been shown to depress blood aceto-acetate levels (Kinsell *et al.*, 1951). The curve of blood aceto-acetate against time may therefore show a dip before rising to a further peak, in excess, sometimes far in excess, of the level produced by simple starvation at a comparable time.

Also the effect of post-operative transfusion of blood or glucose at or immediately after operation was studied: the results suggest that, after the immediate calorie value of the material transfused has been made use of, the post-operative pattern of a high blood aceto-acetate level returns. In three well-defined cases of post-operative ileus no rise in blood aceto-acetate was found. The reason for this may lie, it is suggested, in the temporary ion poisoning of the aceto-CA-kinase enzyme system.

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BOOK REVIEWS

The Haemolytic Anaemias : Congenital and Acquired. By J. V. Dacie. (Pp. x+525. 98 figures. 50s.) London : J. & A. Churchill. 1954.

It is with great pleasure that we welcome a new book which will serve to clarify in the minds of pathologists the many difficult problems which have become so pressing in the last few years. The author's contributions to the subject are well known and are obvious by the many references to the work of his colleagues at the Postgraduate Medical School at Hammersmith and himself.

The haemolytic diseases as exemplified by haemolytic disease of the newborn have been extensively described in the literature, but for reports on other haemolytic processes there has been until now no comprehensive work which could be used as a reference on the subject. The author has presented the main features of the haemolytic anaemias, dividing the congenital abnormality of the erythron from the acquired antibody type of the disease, and he then brings into line those diseases in which the abnormal haemoglobins are the most probable factors in the haemolytic process. In each portion there are case histories which are clear and include a report on the full laboratory investigations, and these are most helpful in focusing the clinical picture of the particular disease. The technical sections are of course excellent, as one would expect from the author of a previous technical book on haematology.

One minor criticism, however, should be noted, and it is hoped that this may be remedied in future editions. It is very trying, even for an experienced haematologist, to see, in a book in which the sizes and shapes of red cells are so carefully discussed and where these features are of such importance, variations in the magnifications of the photomicrographs from ×400 to ×1,000, sometimes on the same page. When the photomicrograph is to demonstrate such things as Pappenheimer bodies this is immaterial, but when it is meant to demonstrate the differences between small spherocytes and large polychromatic reticulocytes it is essential to maintain the same magnification throughout, or else this gives the impression of microcytosis or macrocytosis when it is not intended.

This book is worthy of its place in every pathological laboratory, where it will certainly be frequently consulted, both for its contents and its bibliography.

A. GORDON SIGNY.

Clinical Pathology in General Practice. (Pp. ix+321 ; illustrated. 21s.) London : British Medical Association. 1955.

In this book appear specially commissioned articles on clinical pathology published in the *British Medical Journal* between October, 1953, and July, 1954. As usual with multiple authorship there is no uniformity in the presentation of the articles, nor in their scientific standards. Some of the articles are very clinical and very applied, whereas others describe techniques and methods which are not even used in general hospital laboratories. In fact the general criticism of this book is that it is in no way descriptive of clinical pathology in "general practice," because the editors had clearly not decided in the first instance whether this is a book to bring the general practitioner up to date in the present practice of general pathology, or whether it is intended to describe methods which could be used by the general practitioner. For example, a whole chapter is devoted to necropsy technique, describing simple routine technical details of doing a necropsy which might be considered adequate for the general practitioner, but on the other hand there are details of methods to be used in exhumation, where clearly a specialist would be brought in. On page 113 there is described the spectrographic appearance of pigments in faeces, and on page 260 there is a description of Geiger counters in radioactive iodine tests for thyroid function. This is not in keeping with Professor Bedson's introductory statement on the facilities provided by the National Health Service for general practitioners. It would be preferable to adopt the style of Dr. Mourant on blood grouping, where he describes the *principles* but not the *practice* of blood grouping. There are throughout the book many useful hints to practitioners on the availability of the various tests, their interpretation, and their value.

On the whole the book is easy to read, and if the above criticisms could be met it would become a splended guide to the general practitioner. It is certainly excellent value at 21s.

A. GORDON SIGNY.

The Pathogenesis of Poliomyelitis. By Harold K. Faber. (Pp. xvi+157 ; 16 figures. 36s.) Oxford : Blackwell Scientific Publications. Springfield, Illinois : Charles C. Thomas. 1955.

From many aspects poliomyelitis has been one of the most controversial medical subjects of the past 50 years, particularly with regard to its pathogenesis in man. This has, of course, been linked with differences of opinion concerning the mode of spread and site of entry of the virus into the host. Much has been inferred from the results of experiments in Indian (rhesus) and South East Asian (cynomolgus) monkeys, and a relatively small number of African monkeys and chimpanzees, and this has at times led to considerable confusion because of the presumption that the results represented what occurred in natural infection in man. Even a single strain of virus may behave differently in each of the three species of monkey and chimpanzee, depending on the route of inoculation. In the main, opinion has been divided between those who support a hypothesis that the virus is entirely neurogenic in its affinities, and

those who believe that the virus multiplies in some tissue other than the nervous system, passes thence into the blood stream, and from there may or may not involve the central nervous system. Some workers have altered their views one way or the other as new facts became available as a result of laboratory investigations in either monkeys or man, but Faber has always maintained that the virus is basically neurono-cytotropic and its primary host in the living subject is the nerve cell alone. He believes that

> "The initial invasion of the body tissues ordinarily occurs into the peripheral nerves of the mouth and pharynx, followed by centripetal spread to the regional peripheral ganglia where lies the primary site of virus multiplication. Infection may or may not extend from here to the central nervous system. Virus is excreted into the alimentary lumen by centrifugal axonal spread from infected ganglia."

One of Faber's most recent papers describes widespread invasion of the spinal cord following experimental inoculation into the vertebral artery of monkeys, whereas nerve-borne entry is followed by only restricted distribution, usually in the pons and medulla, which is believed by Faber to form the basis for secondary spread of infection by axonal routes to other parts of the central nervous system. Faber believes this latter process provides a better correlation with the clinical picture at the onset than does viraemia.

This monograph consists of a description of the author's work in monkeys, some of it previously unpublished, and the work of others where it supports his view, all aimed at proving his thesis described above. Although the material makes convincing reading for the most part, one must point out that none of Faber's work has been carried out in chimpanzees, which appear to simulate most closely the behaviour of the virus in man. In fact, in some recent experiments of Bodian, in which the virus was fed to chimpanzees, no virus was recovered immediately before the viraemic stage from the trigeminal and coelic ganglia, but the largest amounts of virus were found in lymphoid tissues, e.g., tonsils and Peyer's patches, as well as in the stools.

F. O. MacCallum.

BROADSHEETS (New Series)

The broadsheets already published are as follows:

(1) Disc Technique for Sensitivity to Antibiotics

(2) Sensitivity of Tubercle Bacilli to Streptomycin

(3) The Detection of Barbiturates in Blood, C.S.F., Urine, and Stomach Contents

(4) The Estimation of Carbon Monoxide in Blood

(5) The Identification of Reducing Substances in Urine by Partition Chromatography

(6) The Paul-Bunnell Test

(7) The Papanicolaou Technique for the Detection of Malignant Cells in Sputum

(8) Investigation of Haemorrhage States with Special Reference to Defects of Coagulation of the Blood.

Broadsheets are obtainable from Dr. W. McMenemy, Secretary, A.C.P., the Maida Vale Hospital for Nervous Diseases, London. Each broadsheet costs 1s. for the first 25 copies, and thereafter 9d.

BOOKS RECEIVED DURING THE YEAR 1955

(Review in a later issue is not precluded by notice here of books received.)

Behandlung rheumatologischer Erkrankungen durch Anästhesie. By Egon Fenz. (Pp. xi+112 ; 18 figures. D.M. 12.) Darmstadt: Verlag von Dr. Dietrich Steinkopff. 1955.

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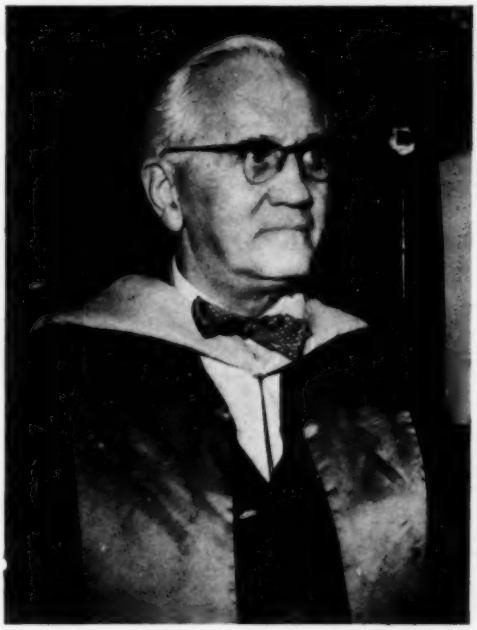
OBITUARY

Sir Alexander Fleming

Sir Alexander Fleming, Professor Emeritus of Bacteriology in the University of London and lately Principal of the Wright-Fleming Institute of Microbiology, died suddenly on March 11 at his home in London with a heart attack at the age of 73. He was buried in St. Paul's Cathedral on March 18.

Alexander Fleming, the son of a farmer, was born near Darvel, in Ayrshire, Scotland, on August 6, 1881, a notable year in the history of bacteriology. His father died when he was 7 years old, and at 13 years of age Alexander went to live with a doctor brother in London, where he continued his education for two or three years by attending the Polytechnic Institute in Regent Street. After four years as a clerk in the City, Fleming in 1902 became a student at St. Mary's Hospital Medical School, where he won the senior entrance scholarship in natural science and practically every class prize and scholarship during his student career. He qualified in 1906, and took his M.B., B.S. of London University in 1908 with honours in physiology, pharmacology, pathology, hygiene, and medicine and a University gold medal.



[*Central Press*

Immediately after qualification Fleming began his association with Almroth Wright in the Inoculation Department at St. Mary's Hospital, and continued to work in the same department for the rest of his life with the exception of an interlude during the first world war. He never ceased to pay tribute to the inspiring leadership of the man whom he regarded as the greatest of British bacteriologists. There is little doubt that in the early years he, like other assistants, and they were a distinguished team, was to a considerable extent the apprentice of Almroth Wright, whose dominant personality and fertile brain directed the general research programme of the Inoculation Department for many years. But even in the early days Fleming showed his ingenuity and originality in devising simple apparatus and techniques for tackling laboratory problems. His first publication in 1908 was a contribution to a symposium in the *Practitioner* on "The Opsonic Method of Vaccine Therapy." Fleming's article, running to 28 pages, was a critical analysis of the opsonic index which had recently been introduced by Almroth Wright as a laboratory indicator of the progress of patients with various infective diseases. Besides a detailed and critical examination of the various components in the opsonic index, Fleming made some interesting and original observations on auto-agglutination of red blood cells in infective conditions, which perhaps heralded the later discovery of the rapid sedimentation rate associated with infection. In the following year a brilliant essay on "Acute Bacterial Infections," which won him the Cheadle Gold Medal, was published in *St. Mary's Hospital Gazette*.

His technical skill was early exemplified by an article published in the *Lancet* in 1909 on a "Simple Method of Serum Diagnosis of Syphilis," some part of which is still used in the Institute. There were further publications on the opsonic index, on the use of salvarsan in the treatment of syphilis (it is noteworthy that Fleming was perhaps the first man to use the new chemotherapy in this country), and on the aetiology and vaccine treatment of acne vulgaris before the first world war directed his attention to wound infection and its control. In 1915 he published a most interesting article on the "Bacteriology of Septic Wounds," and a few years later he showed with Porteous that most wounds become infected with *Streptococcus pyogenes* after admission to hospital. These findings were confirmed and amplified in the second world war. As Wright's disciple he took an active part in the controversy on the physiological versus chemical treatment of wounds, and again his inventiveness in devising apparatus and experiments to test the validity of a hypothesis or statement was very well demonstrated. This work led on to one of his classical experiments, in which he showed that carbolic acid when added to blood in certain concentrations killed the leucocytes but not the patho-

genic germs, so that the antiseptic actually helped rather than hindered bacterial multiplication.

In 1922 came the discovery of lysozyme. Following an observation that his own nasal secretion after a common cold had an inhibitory action on some of the bacteria present in the nose, he showed that this lytic substance was present in many tissues and secretions of the body and was also widely distributed in Nature. But, because it was not particularly active against pathogenic organisms *in vitro*, the medical world did not give it the attention it deserved. His work with lysozyme prepared the way for the epoch-making discovery of penicillin.

Fleming had been studying variation in the staphylococcus, which meant that he was frequently examining colonies of that organism as they appeared on ordinary nutrient agar. As a result of this exposure a mould appeared on the culture medium some days later, and Fleming noticed the unusual phenomenon of the disappearance of staphylococcal colonies around the mould. His first paper describing his work with the active principle of this mould, which he named penicillin, showed that he appreciated the great potentialities of this substance as a new kind of antiseptic, particularly as it had no toxic effect on the phagocytic leucocytes. A few years later he reported that "it has been used on a number of indolent septic wounds and has certainly appeared to be superior to dressings containing potent chemicals."

Following the introduction of penicillin as an antibiotic drug, Fleming devised a number of micro-techniques for its estimation in serum, etc., which became widely used. He was among the first to use phase-contrast microscopy in this country, and with its help he showed that, contrary to a recent claim, flagella were closely associated with bacterial motility. He made innumerable interesting discoveries which have never been published, and just before he died he had been demonstrating to us the effect of different qualities of paper in bringing out the pigment of chromogenic bacteria.

Fleming was a reticent, taciturn, and humble man, with an independent mind, great tenacity of purpose, a tough yet sensitive personality, and abiding loyalty. He was a born naturalist, keenly observant of everything around him, and always ready to devise and test new methods for studying germs in the laboratory or seeds and plants in the garden. He accepted the many honours that were showered upon him modestly and unaffectedly. The simple kind of tribute touched him most, such as a collection of signatures or a letter from a child or from some poor person who had benefited from penicillin. He had been looking forward to working again with opsonin, phagocytes, and lysozyme, for the natural defences of the body were his constant interest. Now, instead, we mourn the loss of a great scientist and a great man.

ROBERT CRUICKSHANK.

Professor H. M. Turnbull

We regret to announce the death on September 29 of Professor H. M. Turnbull, Emeritus Professor of Morbid Anatomy in the University of London. He was an honorary member of the Association of Clinical Pathologists. It is hoped to include an obituary notice in the next issue.

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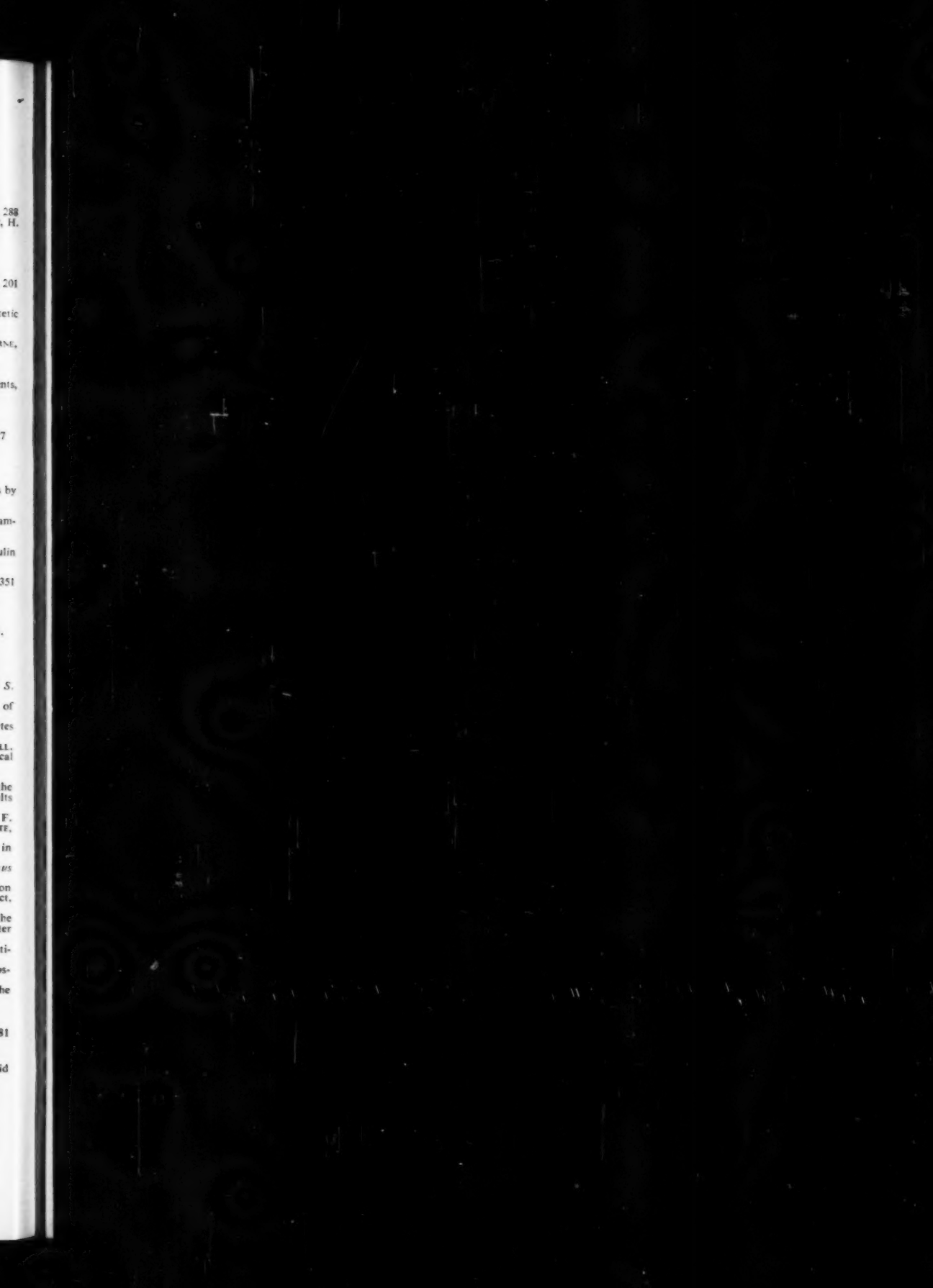
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